Remarks

Claims 1-19 were pending in this application. Of those, claims 11-15 stand rejected and claims 1-10 and 16-19 were withdrawn. With this amendment, claims 1-10, 12-14 and 16-19 are canceled, claims 11 and 15 are amended, and claims 20-35 are added to more particularly point out and distinctly claim the invention.

Applicants respectfully request reconsideration and withdrawal of the current rejections based on the amendments and the following remarks.

Rejections under 35 U.S.C. 112, first paragraph

Claims 11-15 stand rejected under 35 U.S.C. 112, first paragraph for failing to comply with the written description requirement. The PTO alleges that the patent application does not establish that the applicants had possession of the invention for the full scope of the claims with respect to monitoring any disease or disorder, by measuring any interferon producing cells, or where the cells were obtained from any origin. Applicants respectfully request reconsideration and withdrawal of these rejections based on the following discussion.

Applicants note that the claims as amended are directed only to diseases or disorders resulting from HIV infection, and involve measuring pDC2 cells only, in blood or lymphoid tissue only. These methods are disclosed sufficiently in the specification to establish that the inventors had possession of the invention. See, e.g., FIGS. 4-6, page 6, line 10 - page 7, line 2; page 22, line 32 - page 25, line 21; and page 32, line 23 - page 39, line 4.

Regarding the assertion, made at page 4 of the Office Action, that the specification fails to establish a correlation and/or control sample range for pCD2-interferon-producing dendritic cells, which can be used as a reference range to evaluate the progression of HIV-infection, applicants first note that the specification establishes that production of IFN-α is closely correlated with pCD2 cells, such that any measurement of IFN-α would be expected to be a good measurement of the number of pCD2 cells present. See, e.g., page 28, line 33 - page 32, line 7. Additionally, the

establishment of a control or reference range of pDC2 cells for any particular population could be determined without undue experimentation by the skilled artisan, using the methods disclosed in the specification.

Based on the above discussion, applicants assert that the invention described in the claims as amended was described in the instant application sufficiently to establish possession of the invention. Accordingly, withdrawal of the written description rejections is respectfully requested.

Claims 11-15 also stand rejected under 35 U.S.C. 112, first paragraph for failing to comply with the enablement requirement of that paragraph. It is alleged that the claims are not described in the specification in such a way as to enable a skilled artisan to make or use the invention, particularly with regard to monitoring the progression of any disease or disorder, by measuring any interferon producing cells. It is also alleged that the claims are not enabled because they do not establish a control sample range for pDC2 cells.

As discussed above in relation to the written description rejections, the claims as amended are only directed to disorders resulting from HIV infection, and involve measuring pDC2 cells only, in blood or lymphoid tissue only. As such, applicants assert that the claims are fully enabled, since the skilled artisan would understand that the disclosure establishes a correlation between disorders resulting from HIV infection and pDC2 cell occurrence in blood or lymphoid tissue. As previously discussed, the establishment of a control or reference range for any particular population can be effected without undue experimentation by the skilled artisan, simply by quantifying pDC2 cells in a sufficient number (determined by routine statistical methods) of healthy, non-HIV infected individuals. Although subsequent work has established that pDC2 populations decrease with age (Shodell et al., cited at page 8 of the Office Action), the importance of establishing a control range appropriate for the individual tested would have nonetheless been understood at the time of filing.

Regarding the objection that the identification and characterization of NIPC remains the "most significant impairment to studies of IFN-a system in human peripheral

blood" (page 8-9 of the Office Action), applicants note that the NIPC (also known as IPC) are identified in the instant application as pDC2 cells. Methods for the identification of pDC2 cells is described in the specification at page 28, line 33 - page 32 line 7. Thus, the present invention resolves the problems with characterization of IFN producing cells. Thus, the present invention enables the ability to precisely quantify NIPCs (e.g., using cell surface markers characteristic of pDC2 cells). See also F.P. Siegal et al., 1999, Science 284:1835-1837

Applicants also note that the asserted enablement of the claimed methods has been confirmed in Siegal et al., 2001, AIDS 15:1603-1612, and Feldman et al., 2001, Clin. Immunol. 101:201-210 (copies enclosed).

Based on the above discussion, applicants request withdrawal of the enablement rejections.

Rejections under 35 U.S.C. 102(b)

Claims 11-15 stand rejected under 35 U.S.C. 102(b) as being anticipated by Howell et al., 1994, Clin. Immunol. Immunopathol. 71:223-230. Applicants respectfully request reconsideration and withdrawal of these rejections based on the claim amendments and the following discussion.

The claims as amended are directed to methods requiring the measurement of pDC2 cells. The pDC2 cells were identified as a unique cell lineage by Grouard et al., 1997, J. Exp. Med. 185:1101 (copy enclosed). Howell et al. does not describe the measurement of pDC2 cells because Howell et al. did not identify his cells as dendritic cells, or of any particular cell lineage. As Howell says, "Unfortunately, the lack of a known, unique cell surface determinant on the NIPC has made phenotypic (as opposed to functional) enumeration of these cells in the patients impossible." Howell, at page 229, left column. It is precisely this phenotypic identification of the predominant NIPCs as pDC2 cells that is enabled by this invention. Conversely, pDC2 cells had not been identified as interferon producing cells before the instant application. Thus, since

Howell et al. does measure the NIPC as pDC2 cells (a lineage already known at the time of Howell), that work does not anticipate the claimed invention.

In light of the claim amendments and the above discussion, applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. 102(b).

Conclusion

Based on the above discussion, applicants respectfully request withdrawal of all current rejections and passage of the claims to allowance. If there are any minor matters preventing that result, applicants request that Examiner Kaushal contact the undersigned attorney.

Applicants believe that the enclosed check for $950, for a three month extension of time, is all that is required with this response. However, if there are unanticipated fees required to maintain the pendency of this application, the PTO is authorized to withdraw those fees from Deposit Account 01-1785. Overcharges may also be credited to Deposit Account 01-1785.

Respectfully submitted

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Interferon-α generation and immune reconstitution during antiretroviral therapy for human immunodeficiency virus infection

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Objectives: To quantify the effect of HIV infection and HIV-suppressive therapy on interferon-α (IFN-α) production by human blood mononuclear cells; to compare, in parallel, effects on CD4+ T-cell numbers; and to ascertain the relationship of these interferon and CD4 parameters to resistance to opportunistic infections.

Design: Serial studies of 294 unselected patients with HIV infection during therapy, with outcomes analysis.

Methods: Determination of IFN generation by blood mononuclear cells via bioassay, and T-lymphocyte subset analysis via flow cytometry; serial studies of individual patients; linear regression and χ^2 contingency table analysis.

Results: HIV burden is inversely related to interferon-α generation, much as it is to CD4+ T-cell counts. Both of these recover during HIV-suppressive therapy. Reconstitution of IFN-α generation to levels commensurate with protection against opportunistic infection occurs prior to similar restoration of CD4 counts. In the outcomes analyses, such immune reconstitution was associated with protection from recurrent or new opportunistic infection. Conversely, viral suppression without such immunologic recovery was not protective against opportunistic infection.

Conclusions: Rapidly responding IFN-α generating cells appear to participate in resistance to opportunistic intracellular infection. Recovery of IFN-α generation may be an early marker of immune reconstitution in AIDS.

AIDS 2001, **15**:1603–1612

Keywords: Dendritic cells, Th1, cellular immunity, natural immunity, immunocompromised host, plasmacytoid, interferons, T cells, interferon-α, HIV, AIDS, natural interferon-producing cell

Introduction

Interferon-α (IFN-α) generation in response to herpes simplex virus (HSV) progressively declines during the course of HIV infection [1–5]. In a previous study of the natural history of HIV infection, reduced IFN-α generation predicted the development of opportunistic infection (OI) as efficiently as did low CD4+ T-cell counts [2]. At the time of those studies, the cell type producing IFN had been characterized as a low-density, non-T, HLA-DR+ blood mononuclear cell that came to be known as the natural IFN-producing cell (NIPC) [6]. Patients resisted OI if either CD4+ T-cell counts were $> 250 \times 10^6$ cells/l or IFN-α generation was > 300 IU/ml: Kaplan–Meier analysis showed the probability of survival free of OI and death over

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the ensuing 36 months to be > 90% as long as either of these apparently independent immune parameters remained above the cutoff values. Patients having the dual defect, in contrast, had 50% Kaplan–Meier survivals around 10 months [2]. These findings thus suggested an important role for NIPC in the host defense against intracellular infections.

NIPC have recently been shown to be identical to the lineage-negative, CD4+, CD11c-, CD123+, dendritic cell (DC) precursors ('pre-DC2'), normally found at low frequency in blood, and in T-cell regions of lymphoid tissues [1,2,6–13]. This characterization permits IFN-α generation in response to HSV to be considered a functional marker of these cells, which are currently being referred to as plasmacytoid dendritic cells (pDC) [13–16].

We prospectively investigated the relationships among plasma HIV-1 RNA (virus load), IFN-α generation, and numbers of circulating CD3+ CD4+ T cells during treatment of patients with HIV infection in a group of 294 patients with HIV infection followed serially from 1996–1998. Analysis of the kinetics of recovery of the two cell types during antiretroviral therapy suggests an early reappearance of pDC function, which was associated with subsequent avoidance of OI and AIDS-related mortality. This clinical outcomes analysis provides additional evidence, originally observed during the natural history of HIV infection [1,2], that functionally competent pDC play a part in resistance to OI. The data suggest that functional recovery of pDCs may be an early marker of the reconstitution of cellular (Th-1 type) immunity during antiretroviral therapy (ART).

Methods

Study population

Between March 1995 and June 1998, we prospectively studied 294 unselected consecutive patients at all stages of HIV infection. There were 59.7% men and 40.3% women: 30.1% were male homosexuals; 29.5% (mostly female) were heterosexually infected; 28.8% were intravenous drug users; 5.0% were Third World immigrants; 3.3% hemophiliacs; 1.7% transfusional; and 1.3% occupational needle-stick recipients. There were 82 (28%) with AIDS/OI; 22 (7%), AIDS with a non-OI case-defining illness [e.g. lymphoma, wasting; (see footnote, Table 3)], 79 (26%), non-AIDS manifestations of HIV infection (e.g. monodermatomal zoster, oral candidiasis, molluscum contagiosum). Sixty-one percent of the subjects thus had 'symptomatic HIV infection'. One hundred (33%) had symptomless lymphadenopathy and 16 (5%) were HIV seropositive without clinical or physical findings.

Almost all received antiretroviral drugs as they became available. Some had received minimally effective single or two-drug regimens until the introduction of the protease inhibitors. Many of these eventually had failing regimens and multidrug resistance as defined by genotypic analysis. For the purposes of this study of the relationship of virus load to interferon-producing cell function, all of the antiviral regimens were considered together, as clinically, immune recovery has not so far been convincingly linked to the specific antiviral agents brought to bear, so long as viral suppression has been achieved.

Prophylaxis for *Pneumocystis carinii* infection was given for CD4+ T-cell counts $< 280–300 \times 10^6$ cells/l; trimethoprim-sulfamethoxazole was most common, then dapsone-pyrimethamine, and (< 2%) aerosolized pentamidine. *Mycobacterium avium* complex (MAC) prophylaxis was started at CD4 cell counts $< 75–100 \times 10^6$ cells/l unless infection was suspected. This reduced the risk of OI relative to that of our earlier (1981–1985) investigations [2], when only around 60% received prophylaxis for PCP, and none for MAC.

Patients were followed serially, typically every 3 months, or depending upon clinical judgment. HIV load, T-cell subsets and IFN-α generation were determined together. Subjects with CD4+ cell counts under 100×10^6 cells/l were monitored with blood cultures for MAC and serum cryptococcal antigen.

Healthy volunteers (n = 257, 710 data points) determined the reference range for IFN-α generation.

Interferon-α generation

Blood mononuclear cells (MNC), isolated on Ficoll/Hypaque, were cultured overnight with UV-irradiated HSV in microwells [17]. Our earlier studies [1,2] had employed HSV-infected human foreskin fibroblasts as stimulus. Supernatants were assayed for IFN using human foreskin fibroblasts as target cells challenged with vesicular stomatitis virus [17]. The assay sensitivity was usually 2–25 IU/ml. Concentrations below detectibility limits were calculated as one unit below the limit of detection. Previous studies have indicated that the IFN produced is > 99% IFN-α as determined by neutralization with IFN-α-specific antiserum and by IFN-α-specific ELISA assays. Carryover HSV in the culture supernatants does not stimulate detectible IFN production by the indicator fibroblasts in the bioassay [1,2,5,6,10].

Virus load

Ethylene diamine tetra-acetic acid-plasma samples were separated, frozen within 3 h and submitted to commercial laboratories (usually Specialty Laboratories, Santa Monica, California, USA) for quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR)

(Roche Amplicor; Roche Molecular Systems, Inc., Hoffmann-La Roche Inc., Branchburg, New Jersey, USA). Ten triplicate plasma specimens at different disease stages were studied blinded by Specialty Laboratories (courtesy of Dr T. Robins and Dr J. S. Sevall). Mean log virus loads for these were 2.86–5.22 copies/ml, SEMs for individuals were between 0.11–0.02, or 0.4-4% of the log virus load. The lower limit of detectability during this study was 400 copies/ml. Virus loads < 400 copies/ml were calculated as 399 copies/ml, and defined as 'full suppression' of viremia.

T-cell analysis

T-cell subsets were defined by a Centers for Disease Control-certified laboratory via two color immunofluorescence flow cytometry; absolute CD3+CD4+ lymphocyte (CD4+ T-cell) counts were calculated from automated complete blood count and differential counts.

Statistical analysis

Q&A version 4.0 (Symantek, Inc., Cupertino, California, USA) and SPSS for Windows version 5.0.2 (SPSS Inc., Chicago, Illinois, USA) were employed for data management and statistical analysis. Chi-square was used to demonstrate the association between clinical outcomes and measures of immune reconstitution. Scatter plots, correlation coefficients and regression lines with 95% confidence intervals (CI) were generated by SPSS for Windows.

Results

IFN-α generation and CD4+ T-cell counts vary in parallel with viral burden

A total of 1506 determinations of virus load, 1827 of IFN-α generation and 1844 of CD4+ T-cell counts in 294 subjects (mean 5.1 determinations per patient, range 1–20) are summarized in Figs 1a and b. Although CD4+ T-cell counts are standard surrogates for disease status, there is striking scatter in the relationship between CD4+ T cells and viral burden. Very similar scattering is seen for IFN-α generation. The correlation coefficients (for log IFN-α generation, $R = -0.4277$; $n = 1497$; $P < 0.0005$; for log CD4 count, $R = -0.4982$; $n = 1312$; $P < 0.0005$) and the regression line slopes (−0.239 for log IFN-α generation, −0.250 for log CD4 count, Table 1) are comparable: The 95% confidence intervals for the two regression lines showed their slopes to be indistinguishable. The similarities in slopes and correlation coefficients with respect to viral burden from these analyses suggest that the two cell types are either: (1) similarly responsive to changes in viral load, or (2) directly linked biologically.

These pooled data involved multiple, but different numbers of, determinations on individual subjects, some of whom progressed through more than one clinical stage. To analyze possible bias introduced by multiple determinations in some patients, we also performed the analysis using additional selection criteria (Table 1) involving equal numbers of determinations for each subject. The slopes of the respective regression lines and their 95% confidence intervals in these different analyses for IFN generation and CD4 counts (Table 1) were indistinguishable, indicating that no bias had been introduced by using regression lines based on all the data points.

IFN quantitation in the present study did not significantly differ from that of our earlier analyses [2], which used a different standard HSV stimulus (see Methods). Log_{10} IFN-α generation by normals in our previous study [2] was 3.25 ± 1.25 (mean ± 2 SD); in the current study, it was 3.26 ± 0.92 (P = NS).

Reconstitution of IFN-α generation precedes that of CD4+ T-cell counts during therapeutic suppression of plasma viremia

We first looked at 31 of the 82 AIDS/OI patients in whom there was full viral suppression (to < 400 copies/ml) (Fig. 2a and b). Recovery of CD4+ T cells and IFN-α generation occurred at comparable rates.

As previously observed, [2] these AIDS/OI patients also generally exhibited dual deficits of IFN generation and CD4+ T cells, prior to virus-suppressive therapy.

As can be seen from the regression lines, recovery of IFN-α generation during viral suppression to levels associated with OI avoidance [2] precedes the development of OI-protective levels of CD4+ T cells by around 6.3 months (95% confidence interval ~ 3.7–9.6 months). The same regression analysis (not shown) was applied to 68 patients with symptomatic HIV infection who had fully suppressible viremia. In this larger (and initially less immunocompromised) group, the geometric mean IFN-α generation reached protective levels at 2.8 months (95% CI, 0.4–4.5 months), whereas CD4 cell counts did so by 10.0 months (95% CI, 8.4–13.0 months).

To exclude the possible bias of multiple determinations on some individuals, we also generated regression lines using only two, rather than all available time points for each study subject whose viremia was suppressed. The several analyses (Table 2) each showed overlapping, statistically indistinguishable slopes for recovery of the two immune parameters. Thus the earlier recovery of IFN generation appears consistent for the study subjects in this data set.

In addition to regression analysis for the groups of

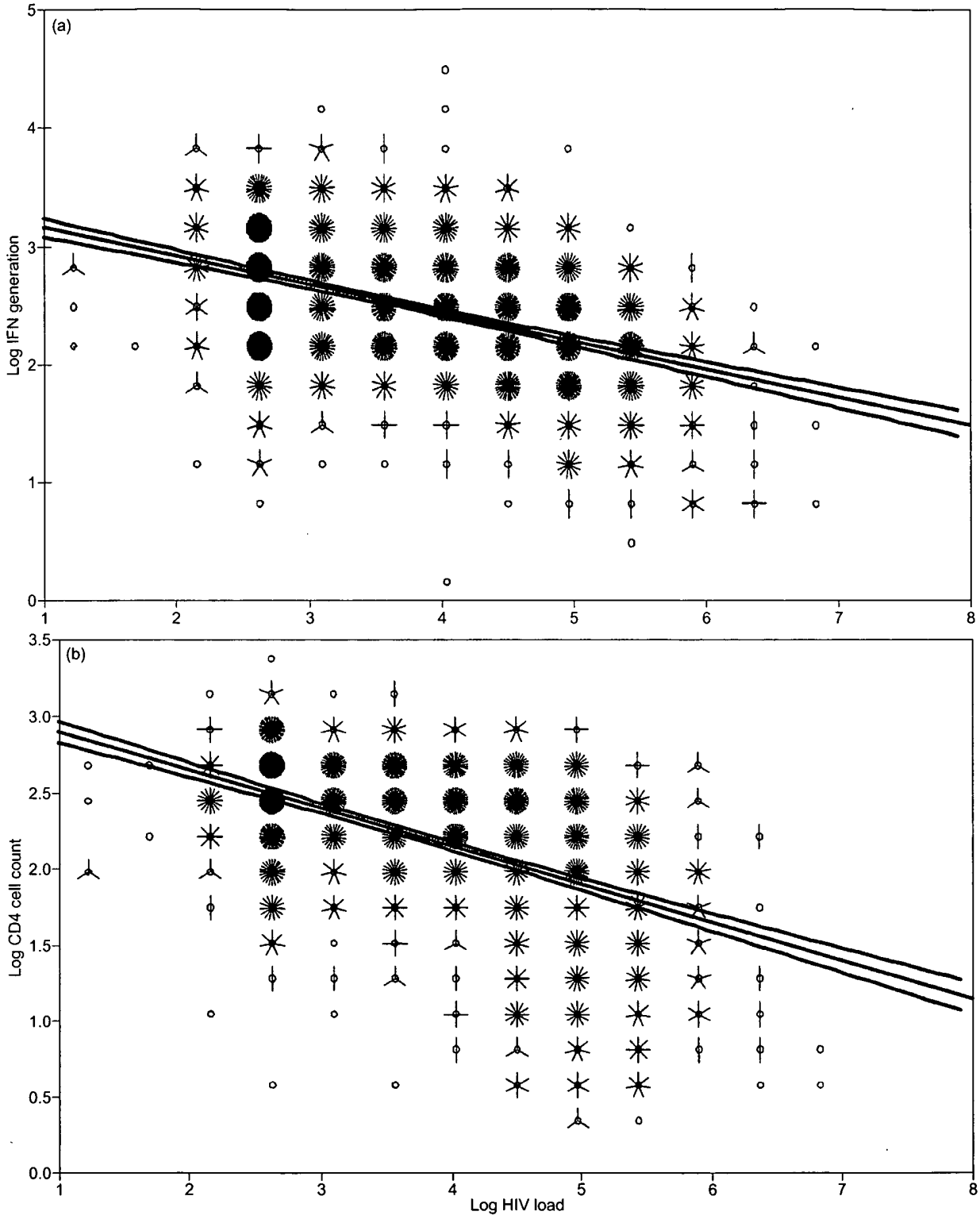

Fig. 1. Relationship of HIV load to (a) interferon (IFN)-α generation and (b) CD4 count. These scatter diagrams represent all the available data points studied for all patients with HIV infection. *X*-axis: log virus load; *y*-axis: (a) log IFN generation and (b) log CD4+ cell count. Individual 'petals' on these 'sunflower' plots show the number of overlapping determinations. Both assays show considerable scatter. The correlation coefficient for log CD4+ cell count (R) = 0.498, and for log IFN generation, $R = 0.428$. The regression line slopes (and their ± 95% confidence intervals) are shown; the slopes do not differ significantly (see text and Table 1).

Table 1. Relationship of HIV burden to interferon-α (IFN-α) generation or CD4+ T-cell counts.

Conditions	Parameter analyzed	Slope of regression line (95% confidence interval)	Correlation coefficient (*R*)
All subjects, all data points			
	IFN-α generation	−0.239 (−0.264 to −0.213)	0.428
	CD4+ T-cell counts	−0.250 (−0.274 to −0.227)	0.498
All subjects, first and last time studied			
	IFN-α generation	−0.233 (−0.281 to −0.184)	0.414
	CD4+ T-cell counts	−0.268 (−0.310 to −0.226)	0.516
All subjects with opportunistic infection, all points while still viremic			
	IFN-α generation	−0.337 (−0.416 to −0.259)	0.417
	CD4+ T-cell counts	−0.239 (−0.318 to −0.160)	0.327
All subjects with opportunistic infection, at highest viral burden			
	IFN-α generation	−0.385 (−0.547 to −0.224)	0.486
	CD4+ T-cell counts	−0.247 (−0.402 to −0.092)	0.356
All symptomatic subjects who were suppressed $T = 0$ and $T =$ last [a]			
	IFN-α generation	−0.215 (−0.281 to −0.150)	0.387
	CD4+ T-cell counts	−0.171 (−0.225 to −0.117)	0.401

[a] $T = 0$, date of last detectable viremia > 400 copies/ml; $T =$ last, last time point at which viremia was still < 400 copies/ml (see Fig. 2 legend).

patients, we looked at the individual serial studies of each of the 82 patients with AIDS/OI. In this individualized analysis (data not shown), IFN generation was found to attain 'protective' levels before CD4 counts did about two-thirds of the time. In the case-by-case analysis, IFN generation was found to recover an average of 5.3 months before CD4 counts. Of note, despite wide scatter of the overall CD4 count and IFN-α generation data (Figs 1 and 2), for individual patients, there was good internal consistency in serial data (e.g. Fig. 3).

We questioned whether the earlier recovery of IFN-α generation could have resulted from an artifact arising from arbitrary cutoff criteria for 'protection' from OI. In our prior study [2], no subject developed OI within 4 months of IFN-α generation $\geq$ 300 IU/ml, or CD4+ T-cell count $\geq 250 \times 10^6$ cells/l. These values were used here to define 'immune reconstitution'. We also re-analyzed the original data [2] selecting the currently more widely accepted value for OI susceptibility of 200×10^6/l CD4+ T cells. Seventeen percent of our earlier patients with CD4+ T-cell counts $> 200 \times 10^6$ cells/l developed an OI. The corresponding 17% protective level for IFN-α generation in the same re-analysis was 150 IU/ml. The use of these lower cutoff values in the regression diagrams did not change the time difference between the two immune parameters, as would have been expected, as the slopes in all analyses are parallel (Table 2).

The patients with AIDS/OI in the current study were statistically indistinguishable from those in our earlier, natural history, study. At their highest viremia [$\log_{10}$ viral burden 5.17 ± 0.878 copies/ml (mean ± 1 SD, n = 75)], which should approximate the natural history of HIV infection, cells from patients in this study generated 2.01 ± 0.70 $\log_{10}$ IU (mean ± 1 SD) IFN-α, whereas the patients studied during 1981–1984, before antiretroviral drugs and virus load measurement, [2] generated 1.72 ± 0.52 $\log_{10}$ IU ($P =$ NS). CD4 counts under the same conditions for patients in this study were $99 \pm 123 \times 10^6$ cells/l (mean ± 1 SD), and in the earlier study, were $187 \pm 208 \times 10^6$ cells/l ($P =$ NS).

Clinical resistance to OI is associated with reconstitution of IFN-α generation

Only 26 of the 82 AIDS/OI patients maintained full viral suppression until the end of the study (Table 3). At the last study determinations, 42 did not achieve 'protective' levels for either IFN-α generation or CD4+ T cells. Deaths from OI, and ongoing or newly developing OI, were restricted to these 42. Of the remaining 40, 15 achieved only the ability to generate IFN-α at $\geq$ 300 IU/ml (e.g. Fig. 3a), three had CD4 counts surpassing 250×10^6 cells/l, but failed to generate protective levels of IFN-α, and 22 recovered both. The association of OI avoidance with immune reconstitution, as herein defined, appears to be highly significant (χ^2 for contingency analysis on Table 3: $P =$ 0.0009).

Three patients had viral suppression to below detectibility for 5–11 months, but nonetheless failed to reconstitute either critical function. Two of these three developed subsequent OI. One patient developed recurrent disseminated *Mycobacterium avium* complex infection (Fig. 3c), and another died of PCP 6 months after his viremia was fully suppressed. In contrast, those 17 patients who had sufficient immune functioning despite persisting viremia did not develop OI or have OI-associated deaths. Those with full viral suppression and reconstitution of both IFN-α generation and CD4+ T cells also remained free of OI. The causes of non-OI-related death are listed in the footnote to Table 3. Thus, OIs and OI-related deaths occurred only when neither IFN-α generation nor CD4 counts had been reconstituted to protective levels.

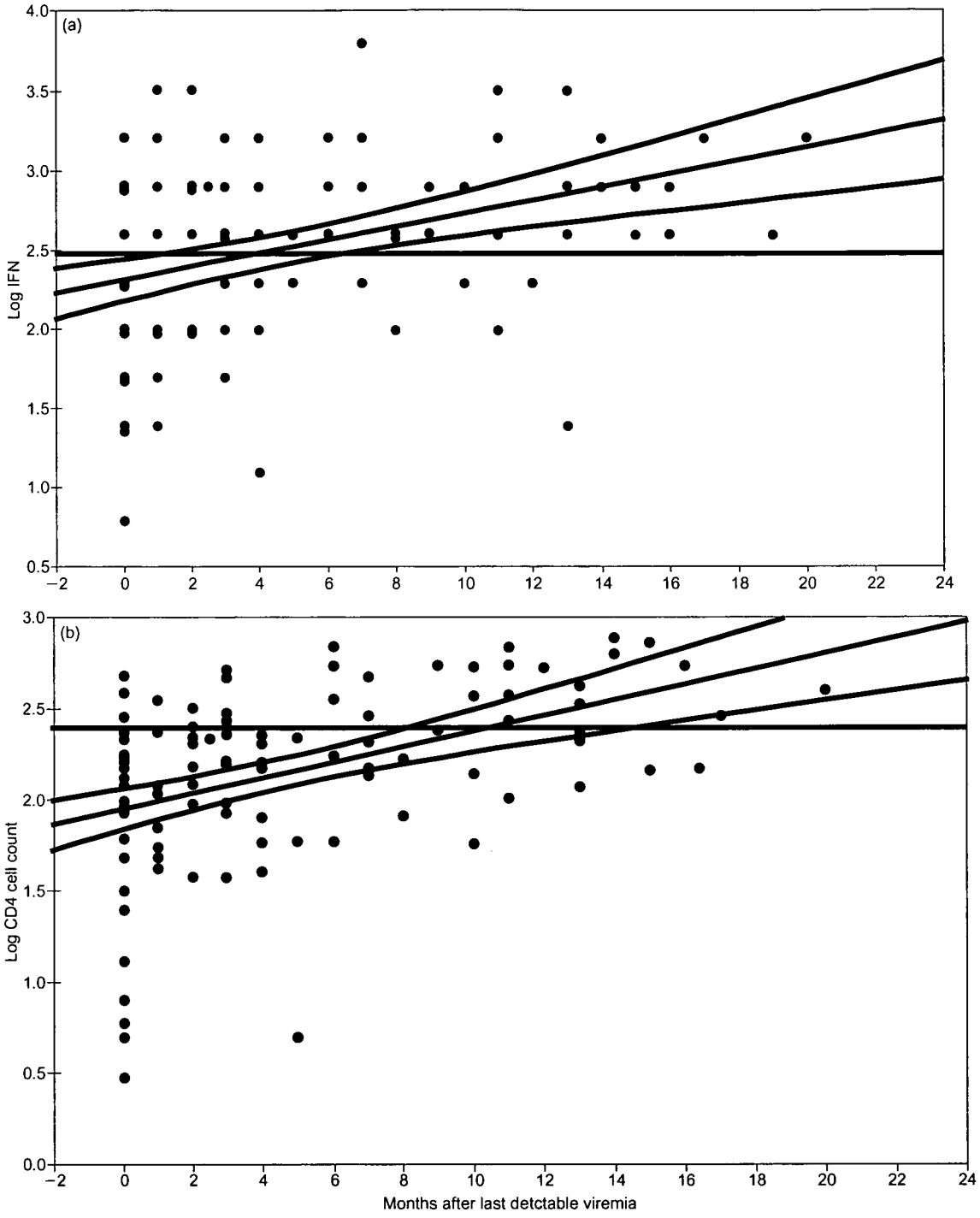

Fig. 2. Time course of recovery of (a) interferon (IFN)-α generation and (b) CD4 counts during therapeutic viral suppression. Scatter plots represent all the available data points for 31 subjects with AIDS/opportunistic infection (OI) and virus loads suppressed to < 400 copies/ml. Most patients contributed more than two time points to these plots (e.g. Fig. 3), while the virus load remained undetectable (See text and Table 2 for other conditions). The horizontal axis represents months after last detectable viremia; time zero is the last date at which virus loads > 400 copies/ml were detected. The vertical axis represents log CD4+ count × 10^6 cells/l or log IU/ml IFN present in supernatants. The horizontal reference lines represent the critically OI-protective levels defined in Siegal et al. [2] (see text), CD4 = 250 × 10^6 cells/l;, IFN = 300 IU/ml. The regression line for IFN-α generation (shown ± 95% confidence interval) crosses the critical reference line at around 4 months, whereas that for CD4 counts crosses at around 10.3 months (see text and Table 2). Reconstitution of IFN-α generation precedes that for CD4+ T-cell counts by about 6.3 months.

Table 2. Reconstitution of interferon (IFN)-α generation or CD4+ T-cell counts after suppression of HIV viremia.

Conditions of analysis	Parameter analyzed	Slope of regression line (95% confidence interval)	Correlation coefficient (*R*)
All subjects with OI, all available data	IFN-α generation	0.0419 (0.0231–0.0607)	0.375
	CD4+ T-cell counts	0.0429 (0.0267–0.0591)	0.453
All subjects with OI, $T = 0$ and $T =$ last[a]	IFN-α generation	0.0538 (0.0290–0.0786)	0.499
	CD4+ T-cell counts	0.0507 (0.0250–0.0763)	0.484
All symptomatic subjects, $T = 0$ and $T =$ last[a]	IFN-α generation	0.0298 (0.0199–0.0398)	0.352
	CD4+ T-cell counts	0.0219 (0.0133–0.0306)	0.328

[a] $T = 0$ is date of last detectable viremia > 400 copies/ml; $T =$ last is last time point at which viremia was still < 400 copies/ml (see Fig. 2 legend).

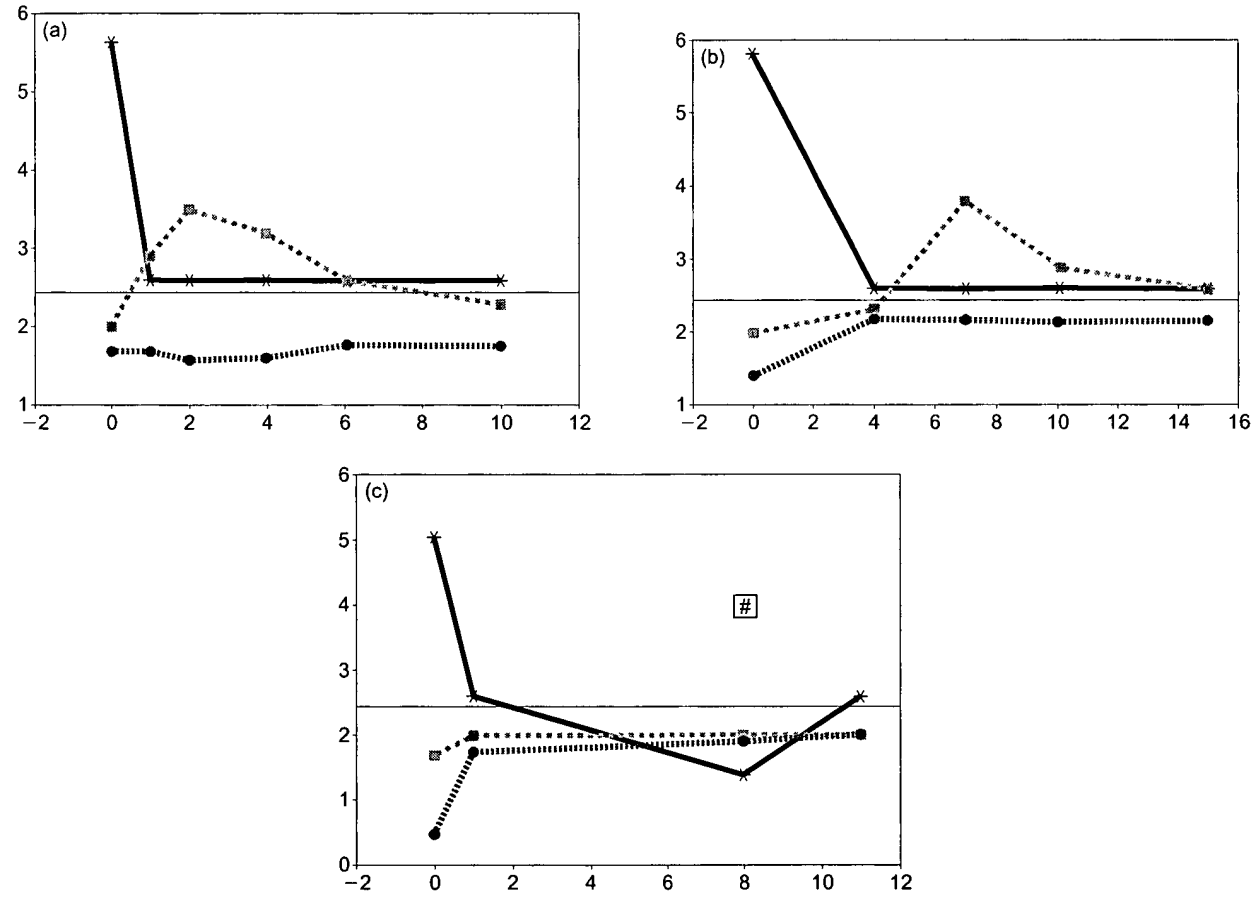

Fig. 3. Different patterns of response to full viral suppression during antiretroviral therapy. Horizontal axis: months from last detectable viremia (> 400 copies/ml). Vertical axis: Log values for viral burden (asterisk), CD4 count (black circle) and interferon (IFN)-α generation (gray square). The horizontal reference line at 2.437 (the log geometric mean of 300 and 250) represents the approximate 'protective' levels for both IFN generation (300 IU/ml) and CD4 counts (250×10^6 cells/l). Panels (a) and (b) typify immune recovery after initiation of active antiretroviral therapy (ART) following an AIDS-defining opportunistic infection. The patient depicted in (a) presented with *Pneumocystis carinii* pneumonia, whereas the subject in (b) presented with active tuberculosis. Viral suppression was associated with gradual recovery of the immune parameters studied in these two subjects. As illustrated by these plots, reconstitution of IFN-α generation most often preceded recovery of CD4+ T cells; opportunistic infections were not observed when IFN-α generation or CD4 count rose above the critical levels represented by the horizontal reference line. Panel (c) illustrates a failure of immune recovery despite effective ART-induced HIV suppression (Table 3), in a patient who presented with disseminated *Micobacterium avium* complex (MAC). The # symbol indicates the point at which blood cultures again became positive for MAC bacteremia, despite MAC-suppressive therapy with four active drugs.

Table 3. Relationship of clinical outcomes to level of viremia and immune reconstitution for 82 patients having a diagnosis of AIDS/opportunistic infection (OI) and treated with antiretroviral drugs.

Viral suppression[a]	Function(s) > cutoff value[b]	IFN generation/ CD4 cell count[c]	No OI mean ± SEM	OI	Died of OI (or other causes)[d]	Total
> 400 copies/ml	neither	82.69 ± 13.28	11	10	16 (2)	39
(4.90 ± 0.13)		47.45 ± 9.15				
< 400 copies/ml	neither	133.33 ± 33.33	1	1	1 (0)	3
(2.60 ± 0.00)		62.67 ± 29.59				
> 400 copies/ml						
(3.98 ± 0.56)	IFN-gen only	470.40 ± 74.46	4	0	0 (1)	5
		91.25 ± 44.54				
(4.26 ± 0.78)	CD4 only	125.00 ± 75.00	2	0	0	2
		310.50 ± 52.50				
(4.16 ± 0.28)	Both	860.00 ± 140.00	9	0	0 (1)	10
		325.22 ± 36.47				
< 400 copies/ml						
(2.29 ± 0.15)	IFN-gen only	680.00 ± 120.00	10	0	0	10
		179.30 ± 14.36				
(2.60)	CD4 only	100	1	0	0	1
		300				
(2.48 ± 0.16)	Both	1033.33 ± 151.42	11	0	0 (1)	12
		496.36 ± 52.90				

[a]Actual $\log_{10}$ HIV-1 load (mean ± SEM $\log_{10}$ copies/ml) by reverse transcriptase-polymerase chain reaction; limits of detectibility levels were calculated for < 40 as 39, or < 400 as 399 copies/ml. [b]Interferon (IFN)-α generation ≥ 300 IU/ml; CD4 count ≥ 250 × 10^6 cells/l, see text and [2].[c]Level of immune reconstitution achieved for the group; numbers derived from last available date of study for each subject in the group; upper number, IFN-α generation/ml; lower number, CD4 count, cells × 10^6/l. [d]Non-OI deaths included: hepatoma, pulmonary embolism, AIDS-dementia complex, central nervous system lymphoma, lung carcinoma. χ^2Pearson, 14 degrees of freedom, value 36.524, $P = 0.00087$ for the contingency table.

Discussion

Studies characterizing immune defects in AIDS have shown abnormalities in virtually all aspects of the immune system [18], including loss of the monocyte-derived CD11c+ dendritic cell [19], lately referred to as DC1 [14,16,20,21]. We have focused on the IFN-α producing cell as its functional loss was strongly implicated in the onset of OI [1,2]. It has now been established that the cell responsible for this IFN generation is the plasmacytoid precursor to type-2 dendritic cells, pDC [12,20,21]. The present paper presents a large data set from prospectively followed HIV-infected patients, from which we analyzed the previously undefined inter-relationships among this pDC function (i.e., IFN-α generation), virus load, and CD4+ T-cell counts. Since the introduction of active ART, clinical and laboratory evidence of immune reconstitution has emerged [22–43]. We therefore analyzed the association of recovering IFN-α generation and CD4+ T cells to resistance to OI during ART.

IFN-α generation in response to HSV bears essentially the same loose relationship to plasma viremia as does the CD4+ T-cell count in our patient population. This parallelism could be attributed either to the direct effects of HIV on two independent cells, or to a biological interaction among them *in vivo*.

During suppression of HIV, IFN-α generation attained the levels we had previously [2] associated with OI resistance several months, on average, prior to re-establishment of protective [2] levels of CD4+ T cells. This difference remains regardless of the protective threshold selected, or the subset of patients studied, since the rates of recovery for the two cell types are consistently indistinguishable. Case-by-case analysis of serial studies of individual subjects reached the same conclusions as did regression analysis for the entire group.

Restoration of protective levels of either IFN-α generation or CD4+ T-cell numbers (the former most of the time) was associated, even in the face of ongoing viremia, with resistance to OI. In contrast, suppression of viremia without such immune reconstitution was not protective: Three of the 82 patients with AIDS/OI had sustained viral suppression but did not achieve critical levels of either IFN generation or CD4 counts; two of these three subjects actually developed OI during the period of follow-up (e.g. Fig. 3c).

IFN-α has recently been shown to bias immature human T cells towards the Th-1 pathway [44–46], leading to enhanced IFN-γ and IL-2 production, both directly and by up-regulating the expression of IL-12 receptors, all being features that are characteristic of the Th1 response. Upon microbial stimulation, blood pDCs appear to migrate via high endothelial venules in lymphoid tissue to T-cell areas, where they can provide IFN-α locally to immature T cells [10–15,47–50]. Strong biasing towards a Th-1 immune response by

these pDC has been observed *in vitro*, whereas immunohistochemical analysis confirms the actual production of type-I IFNs by the pDCs in T-cell areas of lymphoid tissue [11–14]. The progressive losses of IFN-α generation observed during HIV infection [1–5] would be expected to reduce this pathway, ordinarily devoted to Th-1 induction.

As we have shown here, functional regeneration of IFN-α-producing cells during HIV therapy occurs relatively early, heralding protection from OI even when helper T cells remain numerically depleted. Thus, demonstration of improving IFN-α generation could be viewed as an early marker of immunologic recovery during therapy for AIDS.

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Decreased Interferon-α Production in HIV-Infected Patients Correlates with Numerical and Functional Deficiencies in Circulating Type 2 Dendritic Cell Precursors

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Peripheral blood mononuclear cells from patients with human immunodeficiency virus (HIV) infection exhibit a progressively marked decrease in the production of virus-induced interferon (IFN)-α, a finding that correlates with and is highly predictive of disease progression and opportunistic infections. The major IFN-α producing population has recently been defined as the precursor to type 2 dendritic cells (pDC2) or plasmacytoid DC (pDC). Using four-color flow cytometry, we have enumerated the pDC2 vs non-IFN-α producing myeloid DC1 in peripheral blood from HIV-infected patients and healthy controls and related these values to CD4 cell numbers, viral load, and functional activity. The patients had reductions in the numbers of both pDC2 (lin$^-$/HLA-DR$^+$/CD123^{bright}) and DC1 (lin1$^-$/HLA-DR$^+$/CD123^{dim}/CD11c$^+$), both at an absolute level and as a percentage of cells. The decreases were most evident in patients with decreased CD4 levels. Viral load correlated with the functional frequency of the IFN producing cells but not with absolute pDC2 levels. Using intracellular flow cytometric analysis for IFN-α, the patients were demonstrated to have fewer pDC2, as well as a lower percentage of responding cells among those remaining. We conclude that deficient production of IFN-α by pDC2 from HIV-infected patients results from both selective loss of these cells and their qualitative dysfunction. Given the central role of DC, and in particular, DC2, in linking innate and adaptive immune responses, these qualitative and quantitative changes in pDC2 are likely to be key contributors to HIV pathogenesis.

Key Words: **HIV; plasmacytoid dendritic cells; interferon-α; DC2; ELISpot; dendritic cells.**

INTRODUCTION

Our laboratory and others have demonstrated an impaired ability of human immunodeficiency virus (HIV) seropositive individuals to produce interferon-α (IFN-α) in response to virus challenge (1–7). There are two major populations of interferon-α producing cells (IPC) in peripheral blood that respond to viral stimuli *in vitro*, and these are differentially dysregulated in the course of HIV disease (6–9). The first of these is the monocyte that produces IFN-α in response to Sendai virus and, to a lesser extent, other enveloped viruses. The Sendai responsive IPC are a high-frequency population (approximately 1:20 among peripheral blood mononuclear cells (PBMC) from healthy donors) and can produce 0.1 to 0.2 IU IFN-α per cell (6, 9). The second population of cells has been called "natural interferon producing cells" (NIPC) (10, 11). These are present at a much lower frequency than the monocytic IPC (approximately 1:1000 among PBMC from healthy donors as determined by ELISpot analysis) but can produce 1 to 2 IU IFN-α per cell in response to a broad range of enveloped viruses, of which HSV-1 is prototypical (8, 12). Although there was clear evidence that the NIPC belong within the dendritic cell (DC) lineage (13–16), only recently have DC subsets in peripheral blood been clearly described, thus allowing definition of the NIPC as the precursor of the type 2 dendritic cell (pDC2) or plasmacytoid dendritic cell (PDC) (17, 18). The pDC2 are HLA-DR$^+$, lineage (CDs 3, 14, 16, 56, 20)$^-$, CD11c$^-$, CD123 (IL3-Rα)^{bright}. A second population of circulating DC, the DC1, are HLA-DR$^+$, lineage$^-$, CD11c$^+$, and CD123^{dim} (19, 20). This second population of circulating DCs produces little or no IFN-α in response to viral stimulus but was recently reported to produce IFN-α in response to double-stranded RNA (17, 21).

In a large study of patients with different stages of HIV disease, we observed that defects in NIPC function in concert with absolute CD4 cell counts are prog-

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nostic for the onset of opportunistic infections (OI) (2). Conversely, patients with diminished CD4 counts but reasonably normal IFN responses remained free of these OI. In recent studies, we have observed a recovery in the IFN-α production in patients receiving anti-retroviral therapy (22). These findings suggested an important role for NIPC in host protection. Indeed, other studies have suggested that the pDC2, in addition to producing IFN-α, can mature into antigen-presenting cells that, depending on the circumstances, can induce either TH1 or TH2 responses (23–25). In this regard, IFN-α or virus treatment of the pDC2 was found to lead to preferential TH1 cytokine expression (23, 24).

Although both populations of IPC become impaired during HIV disease, the monocyte IPC function declines much later in the course of disease and does not correlate with OI (6, 7). Most recently, we have observed that immune reconstitution in HIV-infected patients on highly active anti-retroviral therapy (HAART) is accompanied by increases in NIPC function as measured by total IFN-α produced in response to HSV, and in fact, this function normalizes before CD4 cell counts and is associated with freedom from OI (22). Together, these studies underscore the importance of failure of components of both the innate (as represented by NIPC function) and the adaptive (as represented by CD4 counts) components of the immune system and, potentially, a breakdown in communication between these components in HIV disease.

Although the functional deficiency of NIPC in HIV infection is well documented, the underlying mechanisms for this deficiency are unknown. For example, decreased IFN production and decreased frequency of NIPC as determined by ELISpot might be accounted for either by a loss of these cells from the peripheral blood or by inability of cells that are present to produce normal levels of IFN-α. The latter possibility is supported by the observation that not only are there fewer total spots in ELISpot assays using blood from HIV-infected patients, but that the spots are also often fainter and smaller, leading to a calculation of significantly less IFN-α production on a per cell basis by cells from the patients (5). Our new insights into the distinct phenotype of the NIPC have now allowed us to enumerate these cells in the peripheral blood using four-color flow cytometry. Furthermore, by utilizing intracellular flow for IFN-α along with surface staining of virally stimulated PBMC, we were able to study the proportion of the pDC2 that respond with IFN-α production. We report that the functional deficiency in NIPC can to a large extent be accounted for by decreased numbers of the pDC2 in peripheral blood of HIV-infected patients. However, even among those pDC2 still present, a lower percentage of the cells respond in the patients than controls, indicating functional deficiency as well.

MATERIALS AND METHODS

Study Population

The HIV seropositive subjects were patients being seen at the AIDS clinics at University Hospital in Newark, New Jersey. This population consists of patients who are at various stages of HIV infection ranging from asymptomatic to those fitting the CDC case definition of AIDS. HIV serology was determined by ELISA and confirmed by Western blot in the Immunovirology Laboratory at University Hospital. Viral load was determined by quantitative PCR by the Molecular Diagnostic Laboratory at New Jersey Medical School using the Roche Amplicor kit. Viral load data are expressed as copies per milliliter of plasma. Healthy staff and students of New Jersey Medical School or University Hospital were used as controls in all experiments.

Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density centrifugation (Lymphoprep, Accurate Chemical and Scientific Co., Westbury, NY) of fresh heparinized peripheral blood obtained with informed consent from healthy volunteers and HIV seropositive patients. The PBMC were resuspended in RPMI 1640 medium containing 10% heat-inactivated FBS (Gemini Biosciences, Woodland Hills, CA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mM Hepes and enumerated electronically with a Coulter Z1 Particle Counter (Coulter Electronics, Inc., Hialeah, FL).

Whole Blood Assay for Determination of DC Subsets

Enumeration of DC in peripheral blood of HIV positive patients and controls was carried out as recommended by Becton Dickinson Immunocytometry Systems (BDIS). Briefly, whole blood was collected in tubes containing EDTA and stained with a lineage FITC cocktail (340546), CD123-PE (340545), anti-HLA-DR PerCP (34764), and CD11c APC (340544), all from Becton Dickinson. Parallel tubes were stained with anti-CD4. Red blood cells were lysed with FACS Lysing Solution, and cells were fixed and analyzed using a FACSCalibur flow cytometer and CellQuest Analysis software (Becton Dickinson). Lineage negative cells were gated, and then analyzed for the expression of HLA-DR and CD123 vs HLA-DR and CD11c. Data are expressed as $CD123^+$ or $CD11c^+$ cells/μl of blood or as a percentage of white blood cells. For CD4

cell levels, data are expressed as $CD4^+$ cells/μl of whole blood.

Virus Preparation

As previously described, HSV-1 strain 2931 and vesicular stomatitis virus were grown and titered on Vero African Green Monkey kidney cells (26).

ELISpot Assays

ELISpot assays were used to determine the frequency of IPC by a simplification of a previously described method (5). PBMC were suspended in RPMI–10% FCS and incubated with HSV-1 at a multiplicity of infection of 1 for 6 to 8 h at 37°C in a 5% CO_2 incubator. Ninety-six-well microtiter plates with mixed cellulose ester membrane bottoms (MultiScreen HA plates, Millipore, Bedford, MA) were coated with ammonium sulfate-precipitated bovine anti-human IFN-α antiserum (Glaxo-Wellcome, Breckenridge, UK) in PBS for at least 5 h at room temperature. The plates were then washed three times with PBS. Stimulated or mock-stimulated PBMC were washed and added undiluted or in serial threefold dilutions to the MultiScreen plates. The cells were then incubated for 12 to 16 h at 37°C and 5% CO_2 to allow production and capture of the IFN-α. The plates were then washed twice with PBS and six times with PBS-T (PBS with 0.05% Tween 20, Sigma) and developed using a murine anti-human IFN-α mAb (293 mAb, provided by Dr. Gunnar Alm, Uppsala, Sweden) diluted in PBS-T–BSA (PBS-T with 3% BSA). This mAb cross-reacts with multiple IFN-α subtypes and was left on the plates for 2 h at RT before being flicked out and the plates were washed as before. This was followed by 1-h incubation at 37°C with goat antiserum to murine IgG conjugated to horseradish peroxidase (HRP, The Jackson Laboratory, Bar Harbor, ME) diluted in PBS-T–BSA. The plates were again washed as described and developed using the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma). These spots were enumerated with the aid of a dissecting microscope and the frequencies of IPC were calculated and expressed as IPC per 10^4 PBMC and as IPC/μl of whole blood.

Intracellular Flow Cytometry for IFN-α with Surface Phenotyping

PBMC were prepared for intracellular detection of IFN-α using a modification of the method described by Milone and Fitzgerald-Bocarsly (16). PBMC at a concentration of 2×10^6 cells/ml were stimulated with HSV-1 strain 2931 at a multiplicity of infection of 1 for 4 h at 37°C in 5% CO_2. Brefeldin A (Sigma) was added to a final concentration of 5 μg/ml and cells were incubated for an additional 2 h. The cells were washed with cold 0.1% BSA (Sigma) in PBS, blocked with 5% heat-inactivated human serum, and stained with the appropriate fluorochrome-conjugated surface cellular marker antibodies (Becton Dickinson or Dako) for 20 min at 4°C. Cells were washed and fixed with 1% paraformaldehyde (Fisher) in PBS overnight at 4°C. The cells were washed twice with 2% FCS (Gemini Biosciences) in PBS and permeabilized with 0.5% saponin (Sigma) in 2% FCS–PBS for 30 min at RT. The cells were pelleted, resuspended in the remaining 50 μl, and incubated with 50 ng of biotinylated 293 mAb to IFN-α for 30 min at room temperature. The cells were washed twice with the permeabilization buffer, resuspended in the remaining volume, and incubated with streptavidin–quantum red (Sigma) for 30 min at room temperature. The washed cells were resuspended in 1% paraformaldehyde in PBS and analyzed using the FACSCalibur flow cytometer with CellQuest Analysis software (Becton Dickinson).

RESULTS

Diminished Peripheral Blood DC1 and pDC2 in Patients with HIV Infection

The frequency and absolute numbers of pDC2 and DC1 in the peripheral blood of 24 healthy vs 70 HIV-infected patients were determined using four-color flow cytometric analysis. A sample analysis using a healthy donor is shown in Fig. 1. DC1 were defined as those lineage-negative, $HLA\text{-}DR^+$, $CD11c^+$, $CD123^{dim}$, whereas pDC2 are $CD123^{bright}$, $CD11c^-$. The data obtained were analyzed and are expressed both as the relative proportion of the two populations (as a percentage of total WBC) and as absolute numbers of the respective populations per microliter of blood. Patient data were further stratified for patients with absolute CD4 counts $<200/\mu$l of whole blood (34 patients) and for those with CD4 counts $\geq 200/\mu$l (36 patients). This stratification of absolute CD4 counts is in keeping with the current definition of AIDS and is slightly different than those originally used by us in our study of the relationship between IFN-α production, CD4 counts, and OI (where we used a CD4 value of 250 for our cut-off) (2). Box plots representing the median, 10th, 25th, 50th, 75th, and 90th percentiles for these data are shown in Figs. 2A and 2B, and the means, standard deviations, and statistical analyses are shown in Table 1. Both subgroups of patients had significantly fewer DC1 and pDC2 than healthy donors, calculated either as a percentage of total WBC or as absolute counts (all P values ≤ 0.002). The patients with the lower CD4 counts had fewer DC of both types than those with CD4 $\geq$ 200; however, for DC1, this failed to reach statistical significance ($P = 0.11$). DC1 in healthy controls were present at approximately a 1.9:1 ratio as compared to pDC2, when calculated as a percentage of WBC and as absolute counts per microliter. Patients with CD4 levels

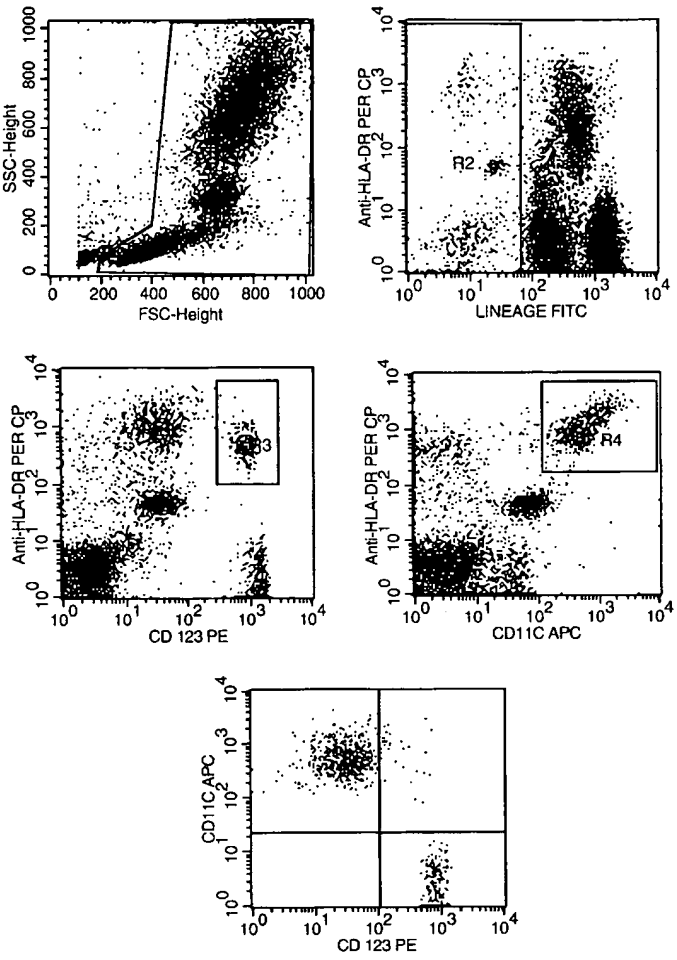

FIG. 1. Flow cytometric analysis for the enumeration of dendritic cells. Whole blood from a healthy donor was stained as described under Materials and Methods. PBMC were gated by forward and side scatter (R1) and the lineage negative fraction (R2) was further analyzed to identify the HLA-DR$^+$, CD123$^+$ (R3) and HLA-DR$^+$, CD11c$^+$ (R4) populations. The bottom panel represents R1 and R2 and (R3 or R4), which shows little overlap between these two markers. R3 detects DC2 and R4 detects DC1 cells.

≥200/μl had a DC1:pDC2 ratio similar to that of the healthy controls, whereas the patients with CD4 counts of <200 had a higher DC1 to pDC2 ratio (2.7), indicating less compromise of the DC1 population than the pDC2 population. Although stratification of the patients by CD4 provided useful grouping, there was no direct correlation between absolute CD4 and pDC2 levels in the patients (Figs. 3A and 3B). Interestingly, for the healthy controls, levels of CD4 and pDC2 did significantly correlate (Fig. 3C).

IPC Frequency vs pDC2 Levels in Patients with HIV Infection

Although the results described above clearly demonstrate reduced circulating pDC2 in the blood of HIV-infected patients, it was important to correlate this with a functional frequency of IPC. This was particularly important since we have shown that at a given time, only a portion of the CD123$^+$ pDC2 from healthy donors respond to HSV stimulation with IFN-α production (Amrute *et al.*, in preparation). To directly compare the frequency of functional IPC vs the presence of the cells in the blood, parallel samples of blood were obtained from patients and analyzed both for IPC frequency by ELISpot analysis and for phenotype as described above. A total of 9 healthy donors, 12 patients with CD4 <200/μl, and 8 patients with CD4 ≥200/μl were compared. There was a significant linear correlation between the numbers of pDC2/μl and HSV-responsive IPC/μl (Fig. 4). For this subset of patients, those with CD4 <200/μl had significantly fewer functional IPC/μl than healthy controls (P = 0.003). whereas those with CD4 ≥200/μl had intermediate levels of functional IPC, which were not significantly different than either the low CD4 group or healthy controls (Table 1).

Correlation of Functional NIPC Frequency with Viral Load

Circulating HIV in plasma (viral load) has been found to be a strong correlate of disease status in infected patients. To determine whether there is a correlation between absolute frequency of circulating DC1 vs pDC2 or with functional IFN-α producing cells (as determined by ELISpot), we plotted these parameters for the 16 patients for whom all of these data points were available. Neither the absolute number of circulating DC1 nor the absolute number of pDC2 was found to significantly correlate with the viral load (Figs. 5A and 5B). In contrast, there was a significant, negative correlation between the numbers of functional IPC/μl blood and the viral load (Fig. 5C, R^2 = 0.365, P = 0.01).

Intracellular Detection of IFN-α in PBMC from HIV Seropositive Donors

It was clear from the data presented above that not all of the pDC2s detected by flow cytometry were HSV responsive IPC as detected by ELISpot. This is consistent with our prior work (Amrute *et al.*, manuscript in preparation) in which we found, using intracellular staining for IFN-α, that between 25 and 60% of CD123$^+$ pDC2 produce IFN-α in response to HSV. To further investigate this in the patient populations, we stimulated patient PBMC samples with HSV, stained them for intracellular IFN-α production, and analyzed them by flow cytometry. Figure 6 shows the flow cytometry data for a representative control and three

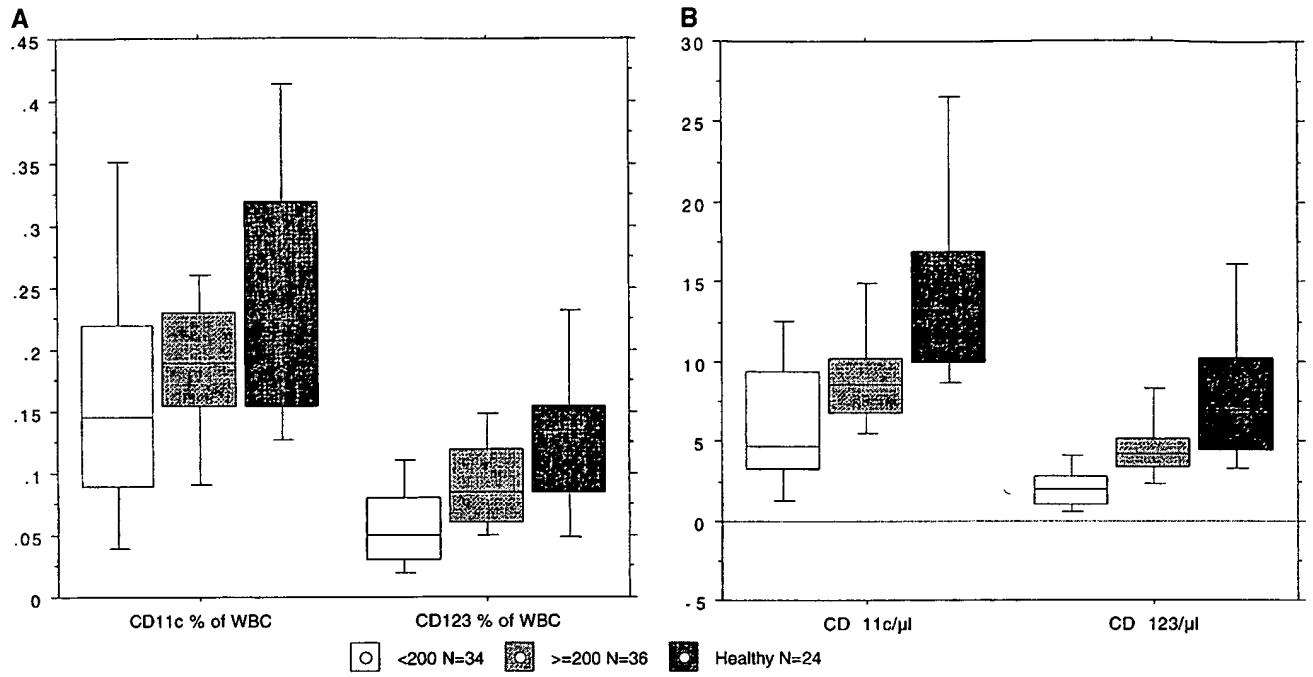

FIG. 2. Distribution of dendritic cell frequencies in HIV seropositive and healthy individuals. Dendritic cell phenotype was determined by whole blood flow cytometric assay as shown in Fig. 1. Box plots showing the median (central line), 25th and 75th percentiles (bottom and top of boxes, respectively), and 10th and 90th percentiles (bottom and top error bars, respectively).

patients as well as other concurrently collected data. Two of the HIV$^+$ donors had both low CD4 levels and low pDC2 levels (Figs. 6E and 6F), whereas a third donor (Fig. 6G) had a CD4 count of 200/μl blood. In agreement with our previous findings, for all of the donors, the functional IPC represent only a portion of the DC2s present. However, the fraction of functional IPC in two of the three patient samples was extremely low (8 and 11% of the pDC2 from the patients were positive for intracellular IFN-α vs 33% for the healthy control (Figs. 6D, 6E, and 6F) and 39% for a third HIV positive donor (Fig. 6G)). Thus, the two patients with the lowest CD4 counts and the highest viral loads had the lower proportion of IFN-α producing pDC2, whereas the third donor had CD4 and pDC2 levels that were more like those of the normal donor, with a viral load below detectable levels.

DISCUSSION

There has been a long-standing observation that deficient IFN-α production in response to viral stimulation by NIPC is associated with susceptibility to infection with opportunistic agents. This correlation

TABLE 1

Data and Statistical Summary of DC1, DC2, CD4, and HSV-Responsive IPC Frequencies in Healthy and HIV Seropositive Patients

	Healthy	All HIV$^+$	HIV$^+$ CD4 < 200	HIV$^+$ CD4 ≥ 200
CD11c % of WBC	0.25 ± 0.02	0.18 ± 0.01*	0.17 ± 0.02*	0.19 ± 0.01*
CD123 % of WBC	0.13 ± 0.01	0.08 ± 0.01*	0.06 ± 0.01*†	0.10 ± 0.01†
CD11c/µl blood	15.6 ± 2.1	7.9 ± 0.6*	6.2 ± 0.8*	9.5 ± 0.8*
CD123/µl blood	8.4 ± 1.2	3.6 ± 0.3*	2.3 ± 0.3*†	4.7 ± 0.4*†
CD4/µl blood	989 ± 94	305 ± 31*	80 ± 11*†	517 ± 29*†
IPC/µl blood	1.6 ± 0.4	0.5 ± 0.1*	0.3 ± 0.1*	0.7 ± 0.1
IPC/10^4 PBMC	4.9 ± 0.8	2.1 ± 0.3*	1.7 ± 0.5*	2.7 ± 0.2*

Note. DC1 (CD11c$^+$), DC2 (CD123$^+$), and CD4 frequencies were determined by flow cytometry as described under Materials and Methods. HSV-responsive IPC frequencies were determined by ELISpot as described under Materials and Methods. The data are expressed as means ± standard error of the mean. * denotes a significant difference from the healthy controls, while † denotes a significant difference between the two patient groups ($P < 0.05$ by ANOVA with Scheffe's F test).

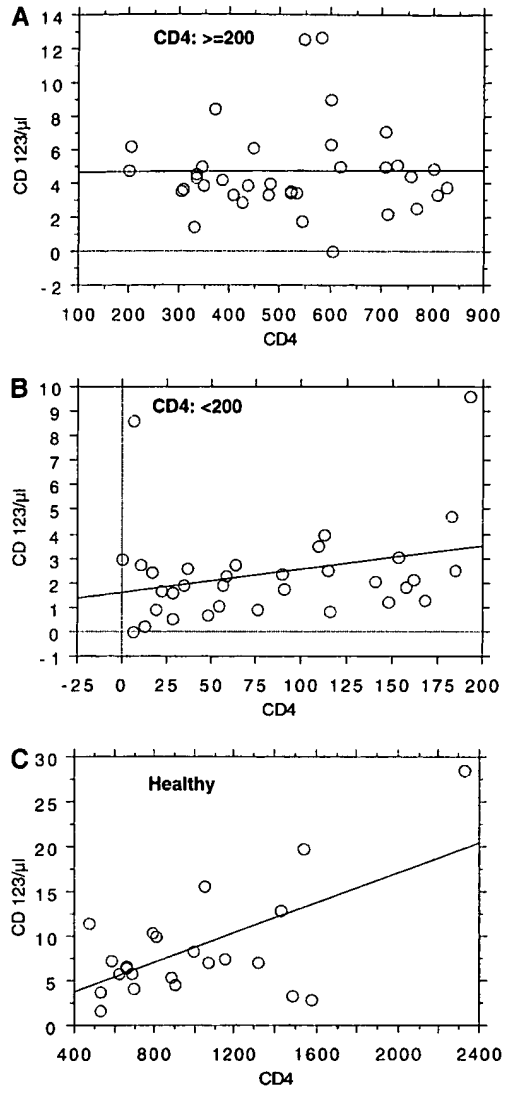

FIG. 3. CD4 and CD123 counts in HIV seropositive patients are not correlated. pDC2 and CD4 frequencies were determined by flow cytometry on whole blood as described under Materials and Methods. The samples were stratified by health and CD4 count (A: CD4 ≥ 200; B: CD4 < 200; C: healthy) and plotted against pDC2 counts. Only the healthy controls showed a significant correlation (P = 0.001) between pDC2 and CD4 counts. R^2 values: healthy controls, 0.384; for CD4 < 200, 0.083; for CD4 ≥ 200, 0.0002.

between deficient IFN production and OI was found to hold for both patients with HIV infection (2) and those with hairy cell leukemia (27). In patients with HIV infection, data in our initial studies were calculated on a per cell basis rather than per microliter of blood, thus indicating that this deficiency was not merely a result of leukopenia (2, 5, 6). Although we were able to clearly demonstrate progressive decreases in both the total IFN produced and the functional frequency of the IFN-α producing cells (as determined by IFN bioassay and IFN-α specific ELISpot assays, respectively), the lack of a distinct defined phenotype of the NIPC made it impossible to correlate functional frequency with the presence or absence of a particular cell; i.e., we were unable to distinguish between a functional deficiency in the NIPC, which would indicate that the NIPC were present but dysfunctional, or their numerical depletion from the peripheral blood. Although the NIPC had long been identified as being within the dendritic lineage, only recently have two distinct DC subsets in the blood been clearly defined: the lineage negative, HLA-DR$^+$, CD11c$^+$, CD123^{dim} DC1 and the lineage negative, HLA-DR$^+$, CD11c$^-$, CD123^{bright} pDC2 (19, 20). Recent evidence suggests that although these cells share overlapping phenotypes and functions, especially in their mature state, they have separate lineages, with the DC1 developing from the myeloid lineage and the DC2 being within the lymphoid lineage, the latter of which express the pTCR-α receptor (28).

Most recently, cell sorting studies have clearly shown the NIPC to reside within the pDC2 population (17, 18), thus allowing us for the first time to carry out studies not only of functional frequency but also of numerical presence in the blood. Using four-color flow cytometry, we have now determined that there is a significant reduction in numbers of both CD11c$^+$/CD123^{dim} DC1 and CD11c$^-$/CD123^{bright} pDC2 in the peripheral blood of patients with HIV infection. These deficiencies were found not only in terms of absolute numbers of DC/µl of blood but also in terms of relative numbers (i.e., proportion of dendritic cell populations among the white blood cells), thus indicating a selective loss of these subpopulations, rather than just generalized leukopenia. When the patients were stratified using absolute CD4 counts (those with CD4 <200/µl and those ≥200/µl), there was a significant difference between the levels of pDC2 in patients with CD4 <200/µl and either the high CD4 group or the healthy control group. These data suggest a concurrent but not necessarily directly linked loss of pDC2 and CD4 cells from the peripheral blood in progressive HIV infection. Indeed, a linear correlation analysis for our patient population indicated no significant correlation between the absolute numbers of CD4 cells and pDC2, but, interestingly, for the healthy individuals, there is a significant correlation between these two cell types. From the data, it appears that the pDC2 numbers may decline to low levels and then remain relatively constant even when the CD4 levels continue to drop. Indeed, our previous data have indicated that very little correlation exists between the functional frequency or total IFN generation in response to HSV and the absolute CD4 levels, indicating that the functional (as opposed to numerical) deficiencies in the NIPC are not directly correlated with the CD4 levels (2, 6, 22).

The concept of both NIPC and CD4 cells independently becoming impaired is corroborated by our ear-

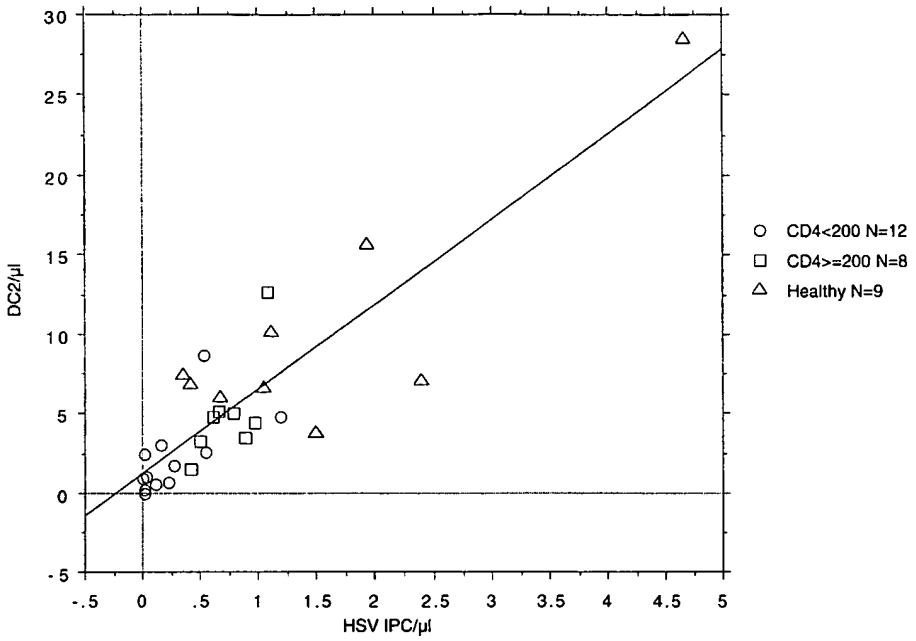

FIG. 4. A correlation exists between pDC2 frequencies and HSV-responsive IFN-α producing cells in healthy and HIV-infected individuals. Parallel samples were analyzed for pDC2 frequencies by intracellular flow cytometry and HSV-responsive IPC frequencies by ELISpot as described under Materials and Methods. The samples were stratified by CD4 count ($\geq$200 or <200) and by health and the results plotted. There was a significant correlation between the two measurements ($P < 0.05$, $R^2 = 0.757$).

lier study in which we compared the production of IFN-α vs the CD4 levels in a large cohort of HIV-infected patients (2). In that study, the data indicated that the susceptibility to opportunistic infections did not occur until there were "critical" compromises in both the NIPC function and the CD4 numbers, with some patients maintaining one or the other function and remaining free of opportunistic infections. In a recent study, we have observed that there is a more rapid reconstitution of the NIPC function (as measured by total IFN response to HSV stimulation) than that of CD4 numbers in HIV-infected patients receiving highly active anti-retroviral therapy, again supporting the concept that the deficiencies in these two populations are not directly linked but, rather, tend to track together with HIV progression. In that study, only total HSV-induced IFN-α was measured and not the absolute numbers of pDC2 or functional NIPC (22). The rate of reappearance of pDC2 in the blood with HAART therapy remains to be examined.

The data obtained by intracellular staining of the IFN-α producing cells demonstrate that not only are there numerical deficiencies in the pDC2 population, but also that the cells remaining are poorly responsive to viral stimulation. In previous studies, we observed using ELISpot assays that the patients not only had a lower frequency of spots, but the spots actually were smaller, which led to a calculation of significantly less IFN-α produced on a per cell basis (5). These data, along with our new intracellular studies, indicate that there is a dysfunction in the pDC2 population even before these cells disappear from the peripheral blood. The mechanisms for the loss in numbers and function of the pDC2 cells from the periphery are unknown. It is possible that HIV infects and kills the pDC2 *in vivo*. This is possible since the pDC2 express CXCR4 and CCR5 and low levels of CD4. Indeed, a recent report indicates that pDC2 can be productively infected with HIV-1 *in vitro* (29). With respect to IFN-α induction in the pDC2 by monocytotropic strains of HIV-1, we have observed that blocking antibodies to CCR5 were incapable of inhibiting the induction of IFN-α, whereas IFN-α induction by these same viruses in monocytes was CCR5-dependent (Dahmani *et al.*, in preparation). While these studies do not directly rule out the possibility of HIV-1 infection of the pDC2, they do suggest that induction of IFN-α by this virus is independent of infection of the pDC2 utilizing CCR5 as a coreceptor. Direct infection studies of the pDC2 with HIV-1 will have to be carried out to fully address this issue. Interestingly, the plasma viral load of HIV was found to significantly correlate with the levels of functional NIPC (as measured by ELISpot) rather than with absolute levels of pDC2. This suggests that high levels of virus, either directly or indirectly, negatively influence the function of the pDC2.

A second possible fate of the pDC2 in patients with HIV infection is that they have tracked from the pe-

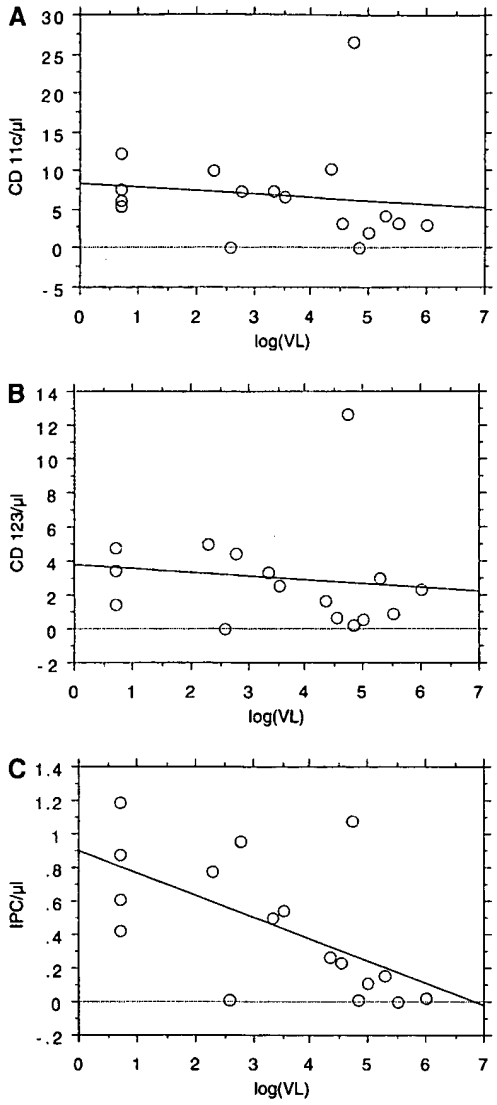

FIG. 5. Viral load does not significantly correlate with DC1 (CD11c) or pDC2 (CD123) frequencies but does negatively correlate with HSV-responsive IPC frequencies. Samples for which parallel DC1, pDC2, and HSV-responsive IPC frequencies (A, B, and C, respectively), as well as for which viral load data were available, were plotted. Neither DC1 nor DC2 frequencies correlated with viral load. HSV-responsive IPC frequencies inversely correlated with viral load ($P = 0.01$, $R^2 = 0.365$).

ripheral blood to the secondary lymphoid organs or tissues. Indeed, peripheral blood pDC2 express L-selectin (CD62L) and are known to traffic to inflamed lymph nodes (18). Studies of patient tissues are planned to address this scenario. The deficient *in vitro* IFN-α production in patients with HIV infection is in contrast to the long-standing observation of high titers of serum IFN-α in HIV patients, which appears early and then again late in the course of disease (30). Our previous results indicated that there is no direct correlation between the presence of serum IFN and *in vitro* generating capacity (31). Zagury and colleagues have reported that the tat protein of HIV-1 is a potent inducer of IFN-α and that this IFN-α is detrimental to the host (32). Although there is no constitutive IFN-α expression by the peripheral blood cells from HIV-1-infected patients, it remains a possibility that the serum IFN-α levels observed in some patients comes from pDC2 that have homed to other areas in the body. Studies of patient tissues are planned to address this scenario.

Two additional possibilities for the functional and numerical deficiencies in the pDC2 are that these cells in HIV-infected patients are unusually susceptible to apoptosis or that they are underproduced by the bone marrow. We have observed that functional IFN-α producing pDC2 are present in the marrow of healthy donors (Amrute *et al.*, in preparation). Parallel studies that would address both the numerical status and the functional status of the pDC2 in HIV-infected patients need to be carried out using marrow of HIV-infected patients.

Our findings of decreased numbers of $CD11C^+$ DC1 in the blood of patients with HIV infection are in agreement with those of Grassi *et al.* (33). These authors proposed that the decreased DC1 in the blood could explain the susceptibility of patients with HIV infection to OI because of the reported role of DC1 in production of IL-12, which leads to induction of TH1 responses. However, more recent data indicate that both DC1 and DC2 are able to induce either TH1 or TH2 responses, depending on the nature of the antigen and conditions for stimulation (18).

Together, the data presented herein clearly demonstrate numerical decreases in both the DC1 and the pDC2 components of peripheral blood of patients with HIV infection. For pDC2, these cells express a functional deficiency in their ability to produce IFN-α as well, especially in patients with advanced HIV infection. Both DC1 and pDC2 are capable of maturing into DC populations that induce TH1 responses. Indeed, IFN-α itself is a TH1 biasing cytokine in humans and can serve as a survival factor for pDC2 (24). Thus, IFN-α, while traditionally thought of as a component of the innate immune system, clearly serves as a link between innate and adaptive immunity. Together, our data help explain the compromise of DC populations and IFN-α producing capacity of the pDC2 and how they might contribute to HIV pathogenesis. We propose that compromises in the numbers and function of pDC2 significantly impair the generation not only of innate immunity (e.g., antiviral effects and augmentation of natural killer cell activity) but also of protective TH1 immune responses. In an earlier study, we demonstrated that susceptibility to opportunistic infections and disease progression did not occur unless both the IFN-α production and the CD4 cell levels were critically compromised (2). Thus, although the CD4 and

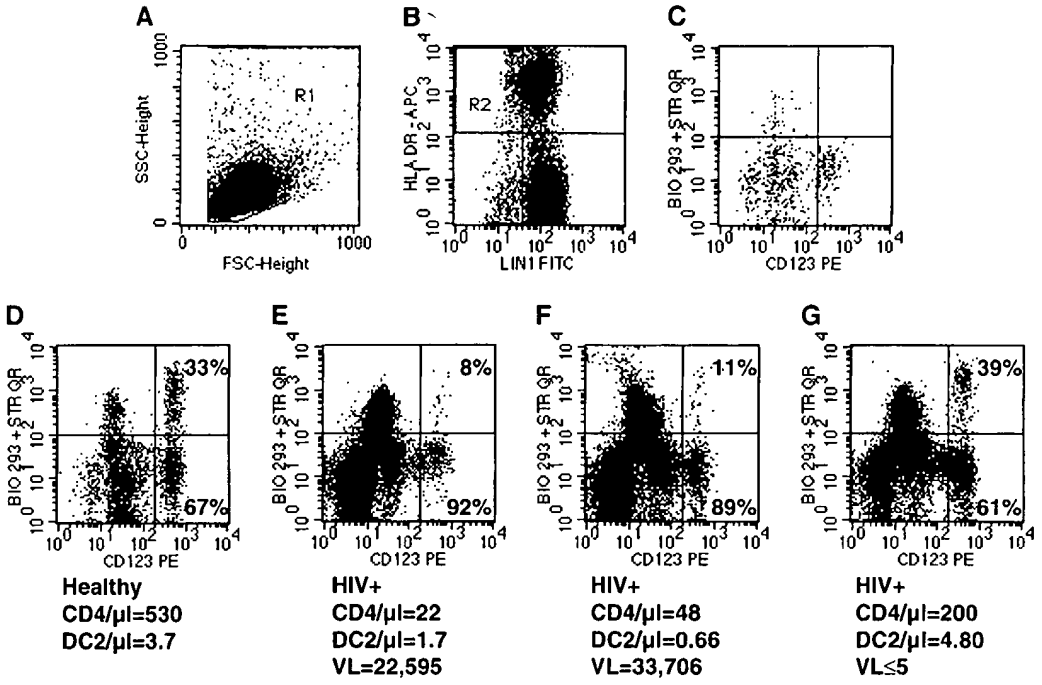

FIG. 6. The proportion and numbers of pDC2 that produce IFN-α in response to HSV challenge are reduced in HIV seropositive patients. PBMC were stimulated for 6 h and then surface stained for HLA-DR, CD123, and lineage markers. Cells were then permeablized and stained for IFN-α. Lineage negative, HLA-DR$^+$ cells were gated and then analyzed for CD123 expression and intracellular staining for IFN-α (A, B, and C; shown for a healthy nonvirally stimulated control). The percentage of IFN producing (upper right-hand quadrant) and nonresponding (lower right-hand quadrant) HLA-DR$^+$, lineage$^-$, CD123$^+$ cells are shown for the healthy control (D) and three HIV$^+$ subjects (E, F, and G.) Numbers of CD4 and DC2 cells are shown for D–G and viral load (VL) for E–G.

pDC2 populations are independently impaired, compromise in both is required for the full immunosuppressive impact to be exerted. Most recently, we have demonstrated that HAART results in improvement of both CD4 numbers and IFN-α production, thus decreasing the susceptibility of the patients to opportunistic infections (22). In addition to providing important information regarding the nature of the deficiency in IFN-α generation in HIV-infected patients, our results further suggest potential clinical value for monitoring levels of circulating dendritic cells in a clinical setting. Although measurement of pDC2 IFN-α producing function requires a laboratory with specialized technology, monitoring of pDC2 numbers can be easily accomplished by four-color flow cytometry and may prove to be a useful clinical surrogate for evaluation of patient immune status in addition to the widely used determinations of CD4 levels and viral load.

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The Enigmatic Plasmacytoid T Cells Develop into Dendritic Cells with Interleukin (IL)-3 and CD40-Ligand

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Summary

A subset of $CD4^+CD11c^-CD3^-$ blood cells was recently shown to develop into dendritic cells when cultured with monocyte conditioned medium. Here, we demonstrate that $CD4^+$ $CD11c^-CD3^-$ cells, isolated from tonsils, correspond to the so-called plasmacytoid T cells, an obscure cell type that has long been observed by pathologists within secondary lymphoid tissues. They express CD45RA, but not markers specific for known lymphoid- or myeloid-derived cell types. They undergo rapid apoptosis in culture, unless rescued by IL-3. Further addition of CD40-ligand results in their differentiation into dendritic cells that express low levels of myeloid antigens CD13 and CD33.

B and T lymphocytes share many similarities, including their developmental programmes, the structures of their antigen receptors, and the requirement for costimulatory molecules for activation. B lymphocytes will finally differentiate into plasma cells that secrete antibody, the key effector molecule of humoral immune responses. Interestingly, pathologists have long observed the presence of plasma cells that express the T cell marker CD4 and lack intracellular immunoglobulins within the T cell zones of human lymph nodes (1–3). These plasma cells were suggested to represent terminally differentiated T plasma cells of unknown secretory functions. Paradoxically, these so called plasmacytoid T cells were found not to express other T cell markers such as CD3 and TCRs (4, 5). They expressed MHC class II antigens, HLADR and HLADQ, and the invariant chain CD74 (6, 7).

The $CD4^+$ plasmacytoid cells were also found in human spleen and thymus from patients with myasthenia gravis (5). These cells were found in increased number in lymph nodes taken from patients suffering from lymphomas, leukemias (8), patients with breast cancer metastases (4), and lymphadenitis (5). In addition, cases of plasmacytoid T cell lymphomas were reported that paradoxically evolved towards myelomonocytic leukemia (9). These data suggest that the $CD4^+$ plasmacytoid cells represent a neglected but important cell type of the immune system. However, the nature and the fate of these plasmacytoid cells has remained unknown.

Here, we report the isolation of plasmacytoid cells from human tonsils. The phenotypical, anatomical and functional characterization indicates that these cells correspond to the $CD4^+CD11c^-$ blood dendritic cell (DC)[1] presursors, that either undergo rapid apoptosis or differentiate into DC upon culture with IL-3 and CD40-ligand.

Materials and Methods

Immunohistological Localization of $CD4^+CD11c^-CD3^-$ Cells. Double stainings on human tonsil sections were performed using mouse IgG1 anti-CD3 (Immunotech, Marseille, France) and anti-CD11c (Dako, Glostrup, Denmark) together with mouse IgG2a anti-CD4 (Innotest, Besancon, France). The binding of mouse IgG1 antibodies was revealed by sheep anti-mouse IgG1 (The Binding Site, Birmingham, UK), followed by mouse anti-alkaline phosphatase-alkaline phosphatase complexes (Dako), the APAAP technique. The binding of mouse IgG2a antibodies was revealed by sheep anti-mouse IgG2a-biotin (The Binding Site), followed by ExtrAvidin-peroxidase (Sigma Chem. Co., St. Louis, MO). Alkaline phosphatase activity was developed by the Fast Blue substrate, whereas peroxidase activity was developed by 3-aminoethylcarbazole.

Purification of $CD4^+CD11c^-$ Lin^- Cells. $CD4^+CD11c^-$ cells were isolated from human tonsils. In brief, tonsils were cut into small pieces and digested for 12 min at 37°C with collagenase IV (1 mg/ml; Sigma) and deoxyribonuclease I (50 KU/ml, Sigma) in RPMI 1640. The cells, pooled from two rounds of tissue digestion, were centrifuged over 50% Percoll (Pharmacia, Uppsala, Sweden) for 20 min at 400 *g*. $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ and $CD20^+$ B cells, and $CD56^+$ NK cells were depleted

[1] *Abbreviations used in this paper:* DC, dendritic cell; GCDC, dendritic cells within germinal centers; HEV, high endothelial veinules; RER, rough endoplasmic reticulum; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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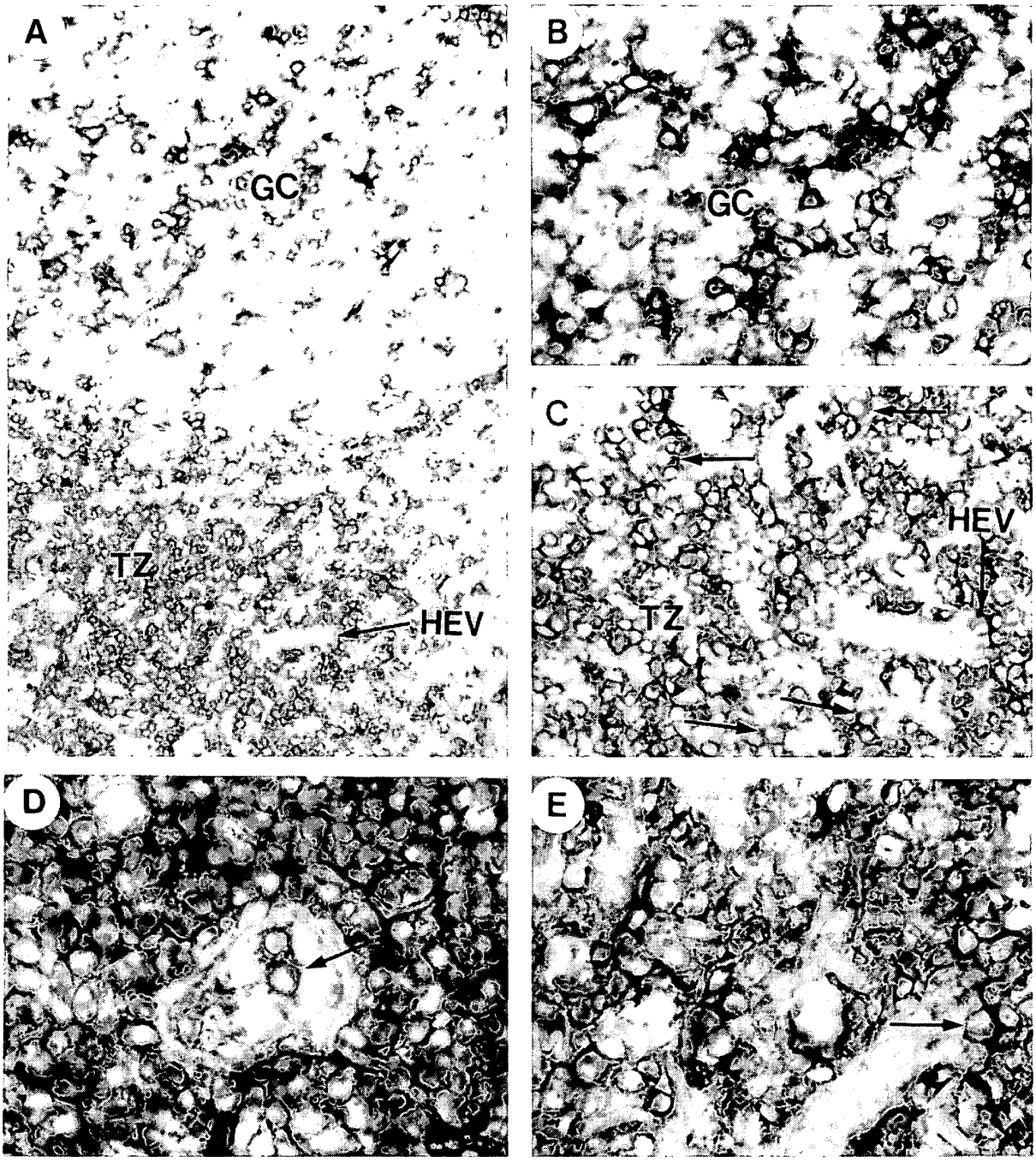

Figure 1. Anatomical localization of $CD4^+CD11c^-CD3^-$ plasmacytoid cells. To distinguish $CD4^+CD11c^-CD3^-$ plasmacytoid cells from $CD4^+CD3^+$ T cells and $CD4^+CD11c^+$ germinal center dendritic cells, double staining with anti-CD3 (*blue*), anti-CD11c (*blue*), and anti-CD4 (*red*) on tonsillar sections was performed. Accordingly, whereas $CD4^+CD3^+$ T cells and $CD4^+CD11c^+$ germinal center dendritic cells should be double stained purple, $CD4^+CD11c^-CD3^-$ plasmacytoid cells should be single stained red. (*A*) shows a germinal center and its adjacent T cell–rich extrafollicular areas containing HEV. Red $CD4^+CD11c^-CD3^-$ plasmacytoid cells can only be found in T cell area but not in germinal center. (*B*) shows that the same germinal center displayed in *A* does not contain red $CD4^+CD11c^-CD3^-$ plasmacytoid cells. (*C*) shows that the same T cell area displayed in *A* contains many red $CD4^+CD11c^-CD3^-$ plasmacytoid cells, located within or around HEV. (*D*) shows a red $CD4^+$ $CD11c^-CD3^-$ plasmacytoid cell within the lumen of HEV. (*E*) shows many red $CD4^+CD11c^-CD3^-$ plasmacytoid cells, one in particular is within the endothelial wall of HEV. Original magnification: (*A*) ×100; (*B, C*) ×200; (*D, E*) ×400.

from the resulting low density cells by immunomagnetic beads (sheep anti-mouse Ig-coated Dynabeads; Dynal, Oslo, Norway). The resulting cells were stained with mouse anti-CD4-PE-Cy5 (Immunotech), anti-CD11c-PE (Becton Dickinson), and a cocktail of FITC-labeled mAbs anti-CD3 and anti-CD34 (Immunotech), anti-CD20, anti-CD57, anti-CD7, anti-CD14, and anti-CD16 (Becton Dickinson), and anti-CD1a (Ortho). Then $CD4^+$ $CD11c^-$ Lin^- cells were isolated by cell sorting. Reanalysis of the sorted cells confirmed a purity of 98%.

Purification of Naive T Cells from Adult Blood. $CD4^+CD45RA^+$ T cells were purified from blood mononuclear cells by immunomagnetic bead depletion using a cocktail of mAbs IOM2 (CD14), ION16 (CD16), ION2 (HLA DR) (Immunotech), NKH1 (CD56), OKT8 (CD8) (Ortho), 4G7 (CD19), UCHL1 (CD45RO), and mAb 89 (CD40). After three rounds of bead depletion, purity of $CD4^+$ T cells was routinely $\geq$96%.

FACS® Analysis of $CD4^+CD11c^-$ Cocktail $FITC^-$-sorted Cells. FITC-labeled mAbs for surface phenotyping of sorted (or cultured) cells were purchased from Immunotech (CD13, CD33, CD34, CD8, CD11b, CD21, CD18, CD54, CD58, and CD38), Becton-Dickinson (CD14, CD2, TCR $\alpha\beta$, MHC class II DR and DQ, CD16, and CD71), Coulter (TCR-$\gamma\delta$), Sigma (CD45, CD44), Dako (CD45RO, CD23, CD11a, and CD11c), PharMingen (CD35), Medarex (CD32). PE-labeled mAbs were purchased from Becton Dickinson (CD80), Immunotech (CD40), and PharMingen (CD86). Cells, incubated with antibody for 15 min at 4°C, were analyzed after one wash with a FACScan® flow cytometer. Negative controls were performed with unrelated murine mAbs (Dako). These negative controls are indicated by filled histograms.

Proliferation Assays and DC Generation in Culture. $CD4^+CD11c^-$ Lin^- cells were cultured with IL-3 in the presence or absence of CD40L fibroblasts (10). All cultures were performed in RPMI 1640 supplemented with 10% of FCS, 2 mM l-glutamine and antibiotics. Cells (1.5×10^4) were seeded in 96-well flat-bottom microtiter plates for the DNA synthesis assay. After 3 d, cells were pulsed with 1 μCi [^{3}H]thymidine for 8 h before harvesting and counting. Tests were carried out in triplicate and standard deviations are indicated with bars. For viable cell recovery estimation, cells were counted by Trypan blue dye exclusion. rhIL-3 or GM-CSF (Schering-Plough Research Institute, Kenilworth, NJ) were used at a saturating concentration of 10 ng/ml (50 U/ml) and 100 ng/ml (200 U/ml), respectively. rhTNFα (Genzyme, Boston, MA) was used at 2.5 ng/ml (50 U/ml). For phenotypic studies, 3×10^5 to 5×10^5 cells were cultured in 48-well flat-bottom plates (5 CD40L fibroblasts for 1 $CD4^+CD11c^-$ cocktail $FITC^-$ cell).

T Cell Proliferation Assay. DC (10 to 5,000) were cultured with 5×10^4 allogeneic blood $CD4^+CD45RA^+$ T cells in round-bottomed 96-well culture plates in RPMI 1640 containing 10% human AB^+ serum. The DC used were generated by culture of (*a*) $CD4^+CD11c^-Lin^-$ cells for 6 d in the presence of IL-3 with or without CD40L transfected fibroblasts; (*b*) blood monocytes for 10 d with GM-CSF plus IL-4 (50 U/ml) (11, 12); and (*c*) $CD34^+$ cells for 12 d with GM-CSF and TNFα (13). After 5 d of DC-T cell cocultures, cells were pulsed with 1 μCi [^{3}H]thymidine for 8 h before harvesting and counting. Tests were carried out in triplicate and standard deviations are indicated with bars.

Electron Microscopic Analysis of $CD4^+CD11c^-Lin^-$ Cells. For electron microscopy, $CD4^+CD11c^-Lin^-$ cells (freshly isolated or cultured cells) were fixed in 2.5% glutaraldehyde plus 0.8% paraformaldehyde in 0.1 M cacodylate buffer and postfixed with an aqueous solution of 1% OsO_4 containing $K_4Fe(CN)_6$. For scanning electron microscopy (SEM), cells were allowed to settle on coverslips precoated with poly-l-lysine, dehydrated in an acetone series, dried by the critical point method (CO_2), and coated with 10 nm gold in sputter device. Observations were made with a SEM 505 instrument (Philips, Eindhoven, Netherlands). For transmission electron microscopy (TEM), the cells were dehydrated in an alcohol series and embedded into epon. Ultrathin sections (~50 nm) were stained with lead citrate and uranyl acetate and studied with a CM 100 instrument (Philips).

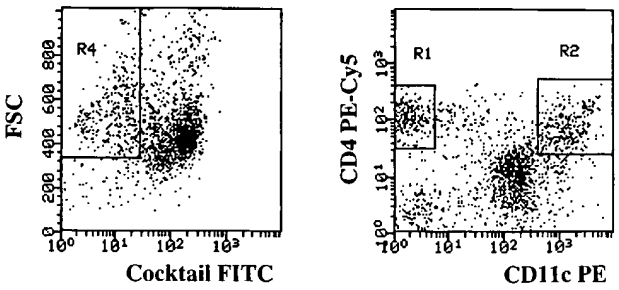

Figure 2. Cell sorting of $CD4^+CD11c^-CD3^-$ plasmacytoid cells. After preparation of tonsillar cells that are negative for CD3, CD14, CD19, CD20, and CD56 (detailed in Materials and Methods), cells were stained with mouse anti-CD4-PE-Cy5 (Immunotech), anti-CD11c-PE (Becton Dickinson), and a cocktail of FITC-labeled mAbs anti-CD3 and CD34 (Immunotech), anti-CD20, anti-CD57, anti-CD7 and anti-CD16 (Becton Dickinson), and anti-CD1a (Ortho). As shown in the left panel, cells were first selected by their cocktail $FITC^-$ (Lin^-). This selected population contains $CD4^+CD11c^+$ germinal center dendritic cells (14) and $CD4^+$ $CD11c^-$ plasmacytoid cells, as shown in the right panel.

Results

Localization and Isolation of Tonsillar Plasmacytoid T Cells. In previous studies, plasmacytoid T cells in situ were shown to express CD4 but to lack lineage markers specific for B cells, T cells, NK cells, monocytes, Langerhans cells, and granulocytes (1–7). They expressed CD11a but not CD11b or CD11c (5). To determine if human tonsils contained $CD4^+$ plasmacytoid T cells, double staining with anti-CD3 (blue) plus anti-CD11c (blue) together with anti-CD4 (red) was performed on tonsil sections. Accordingly, whereas $CD4^+CD3^+$ helper T cells, $CD4^{low}$ $CD11c^+$ interdigitating cells and $CD4^+CD11c^+$ dendritic cells within germinal centers (GCDC) (14) should be double stained purple, $CD4^+CD11c^-CD3^-$ plasmacytoid T cells should be single stained red (Fig. 1). Indeed, red $CD4^+$ $CD11c^-CD3^-$ cells were found in the T cell–rich extrafollicular areas (Fig. 1, *A, C, D, E*) but not within germinal centers (Fig. 1, *A* and *B*), confirming the previous observations in lymph nodes (5). Interestingly, many red $CD4^+$ $CD11c^-CD3^-$ cells were found to be close to or within the high endothelial veinules (HEV) (Fig. 1, *C, D, E*), suggesting that these cells might be blood derived cells that were crossing the HEV. The $CD4^+CD11c^-CD3^-$ cells within the T cell rich areas indeed displayed a plasmacytoid morphology (Fig. 1, *C, D, E*).

According to the in situ phenotype of plasmacytoid T cells, a multistep isolation method was developed includ-

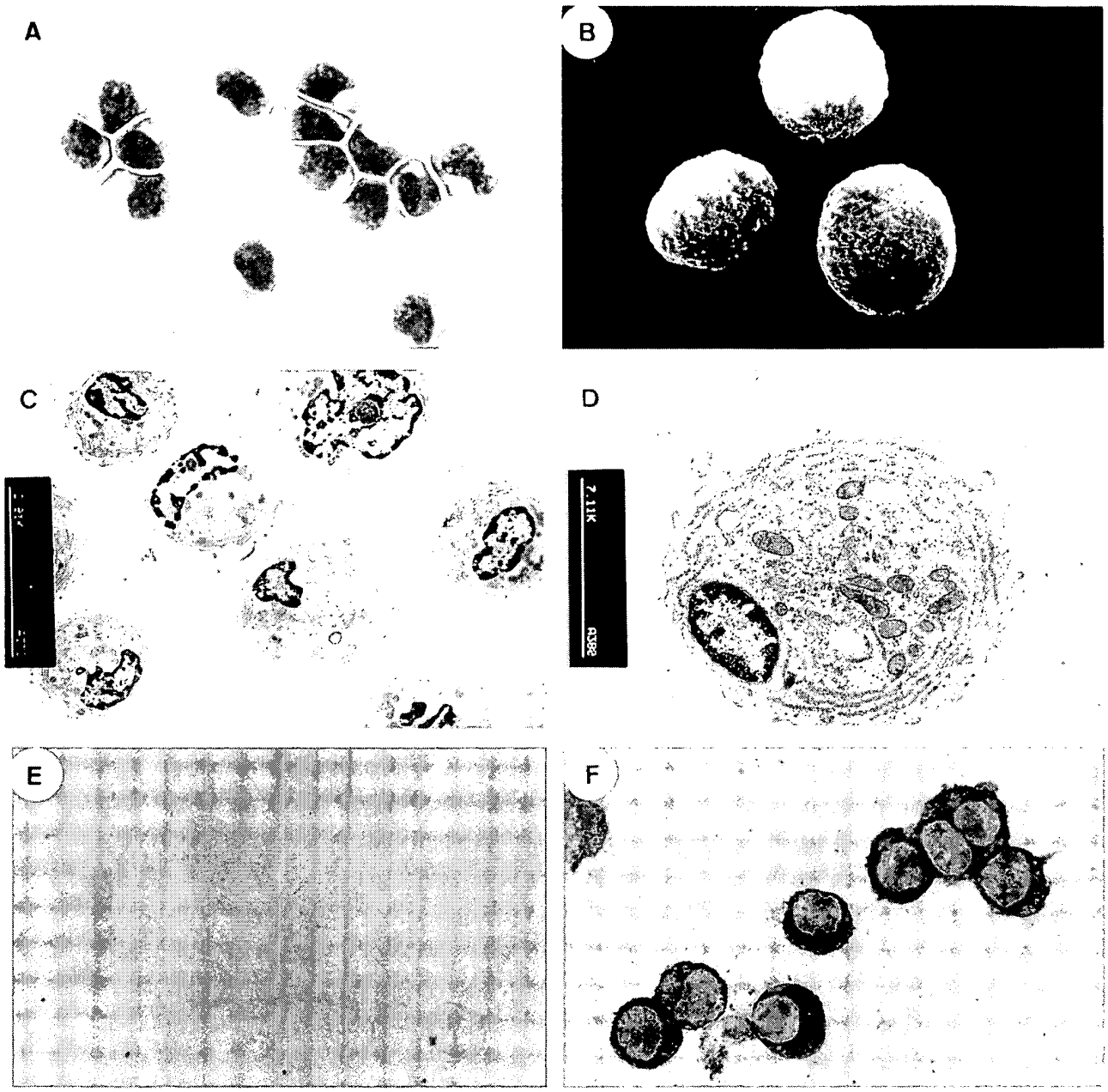

Figure 3. Morphology of freshly isolated $CD4^+CD11c^-CD3^-$ plasmacytoid cells. (*A*) Giemsa staining; (*B*) SEM; (*C*) TEM; (*D*) TEM. (*E*) negative anti-Ig κ+λ light chain staining of $CD4^+CD11c^-Lin^-$ plasmacytoid cells; (*F*) positive anti-Ig κ+λ light chain staining of $CD38^{++}CD20^-$ Ig-secreting plasma cells. Original magnification: (*A*, *F*) ×1,000; (*B*) ×3,500; (*C*) ×2,000; (*D*) ×8,000.

ing: (*a*) centrifugation >50% Percoll; (*b*) magnetic bead depletion of B cells ($CD19^+CD20^+$), T cells ($CD3^+$), monocytes ($CD14^+$) and NK cells ($CD56^+$); (*c*) finally sorting for $CD4^+CD11c^-$ cells that were negative for CD7, CD57, CD20, CD3, CD14, CD1a (Langerhans cells), CD34, and CD16 (Fig. 2). Two $CD4^+Lin^-$ populations were identified (Fig. 3 *A*). The $CD4^+CD11c^+$ population that represents GCDCs has been described earlier (14). On Giemsa staining, freshly sorted $CD4^+CD11c^-$ cells display a plasma cell-like morphology, characterized by an eccentric nucleus, a blue basophilic cytoplasm and pale Golgi zone (Fig. 3 *A*). By scanning electron microscopy, these cells display a smooth round lymphoid morphology and are 8–10 μm in diameter (Fig. 3 *B*). By transmission EM, the cells display nuclei with marginal heterochromatin and cytoplasm containing juxtanuclear Golgi apparatus and parallel arrays of rough endoplasmic reticulum (RER) (Fig. 3, *C* and *D*). However, unlike immunoglobulin containing $CD38^{++}$

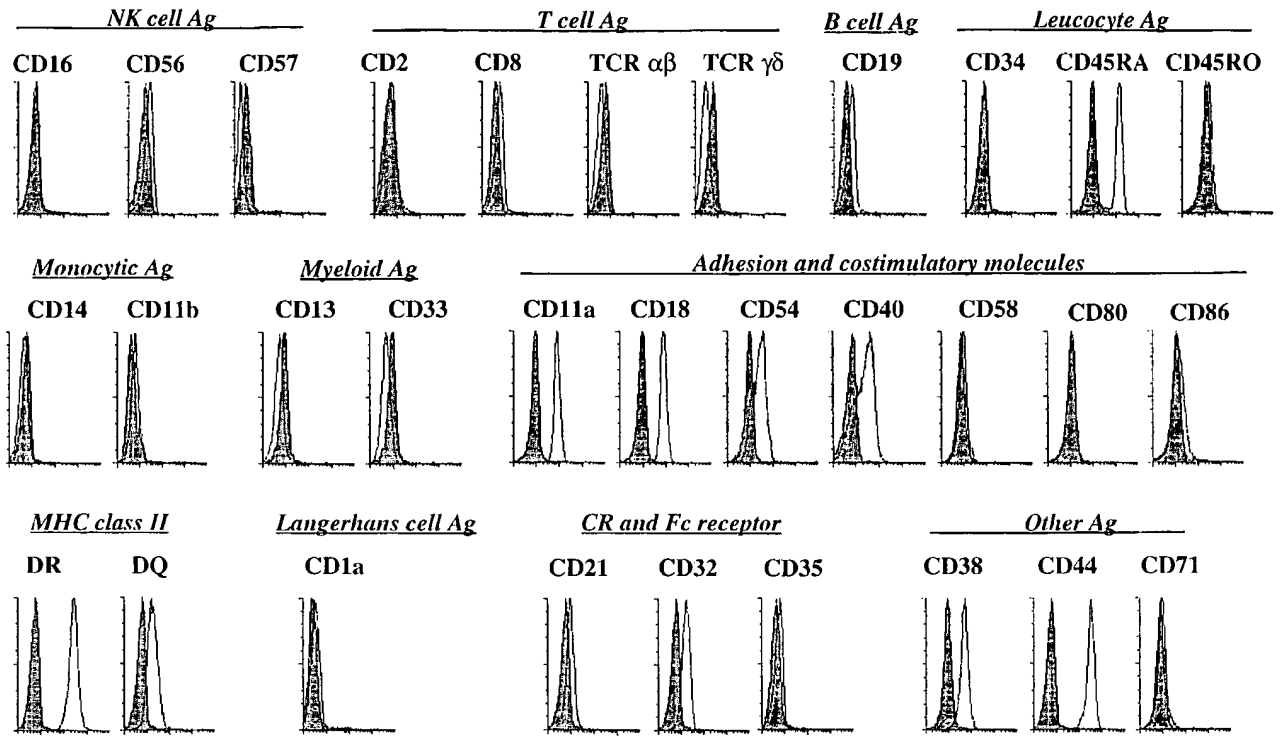

Figure 4. Surface phenotype of isolated $CD4^+CD11c^-CD3^-$ plasmacytoid cells analyzed by flow cytometry. Filled histograms represent isotype matched controls, open histograms represent staining with specific antibodies. Data shown are one representative of six experiments.

$CD20^-$ plasma cells isolated from human tonsils (Fig. 3 *F*), these $CD4^+CD11c^-Lin^-$ plasmacytoid cells did not contain intracellular immunoglobulin (Fig. 3 *E*).

The Phenotype of Isolated $CD4^+CD11c^-Lin^-$ Tonsillar Plasmacytoid Cells. Next, the surface phenotype of isolated plasmacytoid cells was analysed by flow cytometry (Fig. 4). Although $CD4^+CD11c^-$ cells express CD45RA, indicating their hematopoietic origin, they lack all the lineage markers for B cells (CD19, CD21), T cells (CD2, CD3, TCR, CD8), natural killer cells (CD56 and CD57), myeloid cells (CD13 and CD33), monocytes (CD14 and CD11b), granulocytes (CD16 and CD35), and Langerhans cells (CD1a). However, they express MHC class II antigens and the adhesion molecules CD11a, CD18, CD44, and CD54 (but not CD58). They also express low levels of CD40 and CD38. They do not express detectable levels of B7.1/CD80 and B7.2/CD86. This phenotypic profile fits well with that of the in situ plasmacytoid T cells characterized by immunohistochemistry (5). In addition, the phenotype of these tonsillar $CD4^+CD11c^-Lin^-$ plasmacytoid cells is similar to that of the blood $CD4^+CD11c^-Lin^-$ cells, that differentiate into dendritic cells when cultured with monocyte-conditioned medium (15).

Plasmacytoid Cells Die Rapidly by Apoptosis when Cultured in Medium unless Rescued by IL-3. The nature of these plasmacytoid cells was further analyzed by cell culture with different cytokines. Strikingly, these cells undergo rapid spontaneous apoptosis in culture, resulting in 40% of cells displaying apoptotic figures at 4 h (Fig. 5, *B* and *D*) and >90% at 16 h (Fig. 5 *F*). Among a wide range of tested cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, M-CSF, GM-CSF, SCF) and CD40-ligand, IL-3 alone was found to completely block the apoptotic cell death of plasmacytoid cells (Fig. 5, *A* and *C*). In contrast to cells cultured in medium alone, cells cultured with IL-3 rapidly formed aggregates within 4 h (Fig. 5 *A*) and few cells displayed apoptotic figures (Fig. 5 *C*). During the next 6 d of culture with IL-3, >90% of cells remained viable (Fig. 6 *A*) and displayed: (*a*) pseudopods after 16 h of culture (Fig. 5 *E*); (*b*) blastic nuclei and mitotic figures after 3 d of culture (Fig. 5 *G*); and (*c*) veiled morphology after 6 d of culture (Fig. 5 *H*). The $CD4^+$ plasmacytoid cells cultured for 3 d in the presence of IL-3 incorporated [^{3}H]thymidine (Fig. 6 *B*), a finding consistent with the appearance of mitotic figures at this stage. Two cytokine combinations (GM-CSF+TNFα and GM-CSF+IL-4) that, respectively, induce $CD34^+$ progenitor cells (13) and blood monocytes (11) to become DC did not have significant survival (not shown) and proliferation (Fig. 6 *B*) effects on plasmacytoid cells. CD40-ligand, a key survival and activation signal for several cell types (16–18), including B cells (19, 20) and DC (21), did not interfere with the IL-3–dependent survival of plasmacytoid cells, but inhibited the IL-3–dependent [^{3}H]thymidine uptake (Fig. 6 *B*).

CD40-ligand Together with IL-3 Promotes the Differentiation of Plasmacytoid Cells into DC, that Express Little CD13, CD33, and CD1a. As described above, CD40-ligand did not affect the survival of plasmacytoid cells and consistently

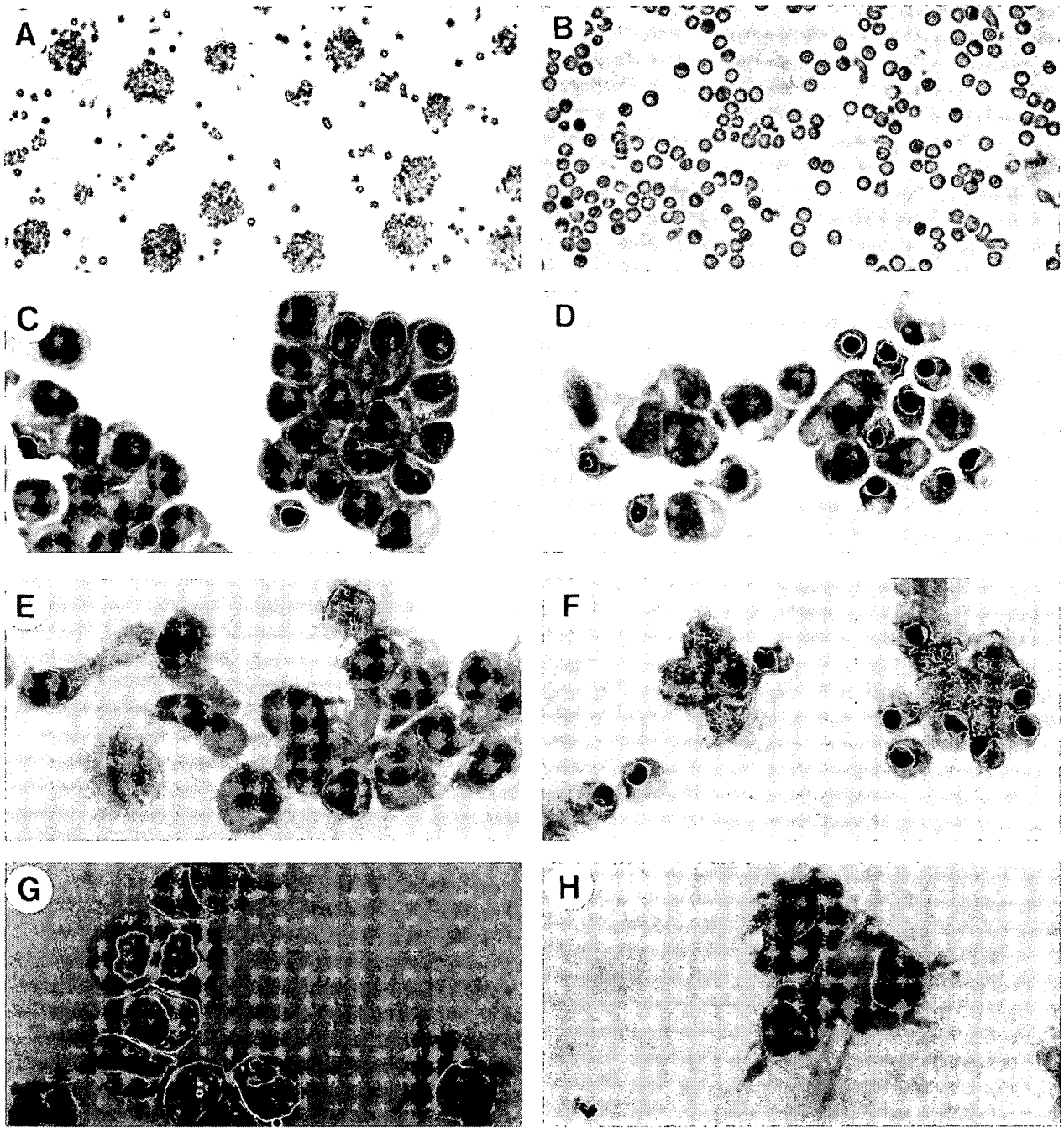

Figure 5. $CD4^+CD11c^-CD3^-$ plasmacytoid cells undergo rapid apoptosis when cultured in medium, unless rescued by IL-3. (*A*) Many cell clusters at 4 h of culture with IL-3; (*B*) Dispersed cells at 4 h of culture in medium alone; (*C*) Few apoptotic cells within clusters at 4 h of culture with IL-3; (*D*) Many apoptotic cells at 4 h of culture in medium alone; (*E*) Cells display pseudopods at 16 h of culture with IL-3; (*F*). All cells are dead at 16 h of culture in medium; (*G*) Mitotic figures at 3 d of culture with IL-3; (*H*) cells with pseudopods at 6 d of culture with IL-3. Data shown are one representative of six experiments. Original magnification: (*B*) ×200.

blocked the [^{3}H]thymidine incorporation induced by IL-3. Most strikingly, CD40-ligand induced the development of long dendrites on these plasmacytoid cells in the presence of IL-3. Fig. 7 *A* shows that after 3 and 6 d of culture, many clusters of cells with long dendrites appeared. This culture morphology was similar to that of dendritic cells generated from $CD34^+$ progenitor cells cultured with GM-CSF+TNFα (13). Indeed, Giemsa staining of cytospin preparation of plasmacytoid cells cultured for 6 d with IL-3 and CD40-ligand revealed a typical mature dendritic cell morphology (Fig. 7 *B*). These observations were further confirmed by TEM and SEM (Fig. 7, *C* and *D*). The cells

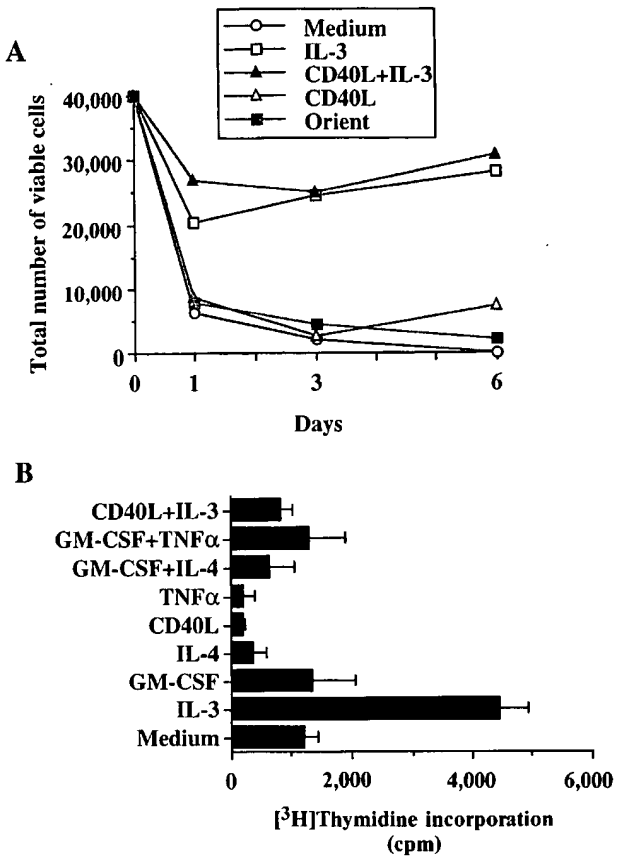

Figure 6. IL-3 maintains the viability of $CD4^+CD11c^-CD3^-$ plasmacytoid cells for up to 6 d. (*A*) Total numbers of viable cells at day 1, day 3, and day 6 of culture with medium (*open circles*), IL-3 (*open squares*), CD40-ligand transfected L cells (*open triangles*), CD40-ligand negative L cells = ORIENT (*solid squares*) and CD40-ligand transfected L cells plus IL-3 (*solid triangles*). (*B*) IL-3 induces [^{3}H]thymidine incorporation by plasmacytoid cells at day 3 of culture. Data shown are one representative of four experiments.

cultured with CD40-ligand and IL-3 developed large dendrites (like spines of hedgehog) (Fig. 7 *C*) and did not have a trace of RER (Fig. 7 *D*).

The phenotype of plasmacytoid cells cultured for 6 d with IL-3 or with IL-3+CD40-ligand (Fig. 8) was subsequently determined by flow cytometry. With IL-3, the adhesion molecule CD58 and the costimulatory molecules B7.1/CD80 and B7.2/CD86 were induced, and the adhesion molecule CD54, the costimulatory molecule CD40 and MHC class II DR were upregulated. CD40-ligand further upregulated the expression of the above molecules. Antigens specifically expressed by myeloid cells (CD13, CD33, CD11b, CD14, CD35), B cells (CD21), Langerhans cells (CD1a), NK cells (CD16), or activated cells (CD25 and CD45RO) were not induced under these two culture conditions. CD11c, a marker highly expressed by different subsets of DC appeared to be induced on a fraction of cells.

Dendritic Cells Generated from $CD4^+$ Plasmacytoid Cells Display Poor Phagocytosis or Endocytosis Capacities but Strongly Stimulate Allogeneic $CD4^+CD45RA^+$ Naive T Cells. To test the functional properties of DCs generated from plasmacytoid cells cultured with IL-3 or IL-3+CD40-ligand, their antigen processing and presentation capacities were analyzed. In contrast to DC generated from monocytes with GM-CSF+IL-4, DC derived from plasmacytoid cells after 6 d of cultures either with IL-3 alone or with IL-3 plus CD40-ligand did not uptake FITC-dextran as analyzed by FACS® (Fig. 9). In addition, they displayed a poor ability to phagocytose FITC-latex beads as analyzed by confocal microscopy and electron microscopy (not shown). Moreover, fresh isolated $CD4^+$ plasmacytoid cells also failed to uptake FITC-dextran during a 40-min culture with FITC-dextran (not shown). However, DC derived from plasmacytoid cells stimulated allogeneic $CD4^+CD45RA^+$ naive T cells and were at least as potent as monocyte-derived DC or $CD34^+$ progenitor cell-derived DC (Fig. 10). The cells with pseudopods generated from plasmacytoid cells after 6 d of culture with IL-3 also significantly stimulated the proliferation of $CD4^+CD45RA^+$ naive T cells (Fig. 10).

Discussion

The experiments described in this article demonstrate that the plasmacytoid T cells, which have long been observed in the T cell areas of peripheral lymphoid tissues, correspond to $CD4^+CD11c^-$ blood DC precursors (15). These cells display six striking features: (*a*) they have a plasmacytoid morphology, characterized by well developed RER and Golgi apparatus, which would normally suggest a terminally differentiated cell type with an active secretory function; (*b*) they die very rapidly by apoptosis in simple culture medium, consistent with the previous report that nuclear pyknosis and tingible body macrophages were found within clusters of plasmacytoid T cells in situ (5) and suggesting a rapid cell turn over rate; (*c*) they are found either close to the HEV or within the lumen of HEV; this, together with the identification of $CD4^+CD11c^-$ blood DC precursors (15), indicates that the tissue plasmacytoid DC precursors are blood derived; (*d*) IL-3 represents the only cytokine tested so far that maintains their survival and promotes their proliferation; (*e*) CD40-ligand promotes their differentiation into mature DC; (*f*) the plasmacytoid cell derived DC display features similar to a subset of tonsillar interdigitating cells (Björck, P., and Y.-J. Liu, unpublished observation), including low expression of myeloid antigens CD13 and CD33 and poor ability to uptake soluble antigens or to phagocytose particles.

DC represent an heterogeneous population of hematopoietic-derived cells that display potent ability to prime naive T cells and to stimulate memory T cells (22). The past few years have witnessed remarkable advances in the isolation of DC precursors and in the definition of culture conditions allowing the generation of DCs (23, 24). DCs have been generated from early hematopoietic progenitors that can be isolated from human and mouse bone marrow (25–28), thymus (29–32), or human blood/cord blood (12, 13, 33, 34). Hematopoietic progenitors isolated from bone marrow were shown to have the potential of becoming dendritic cells as well as granulocytes, monocytes, and mac-

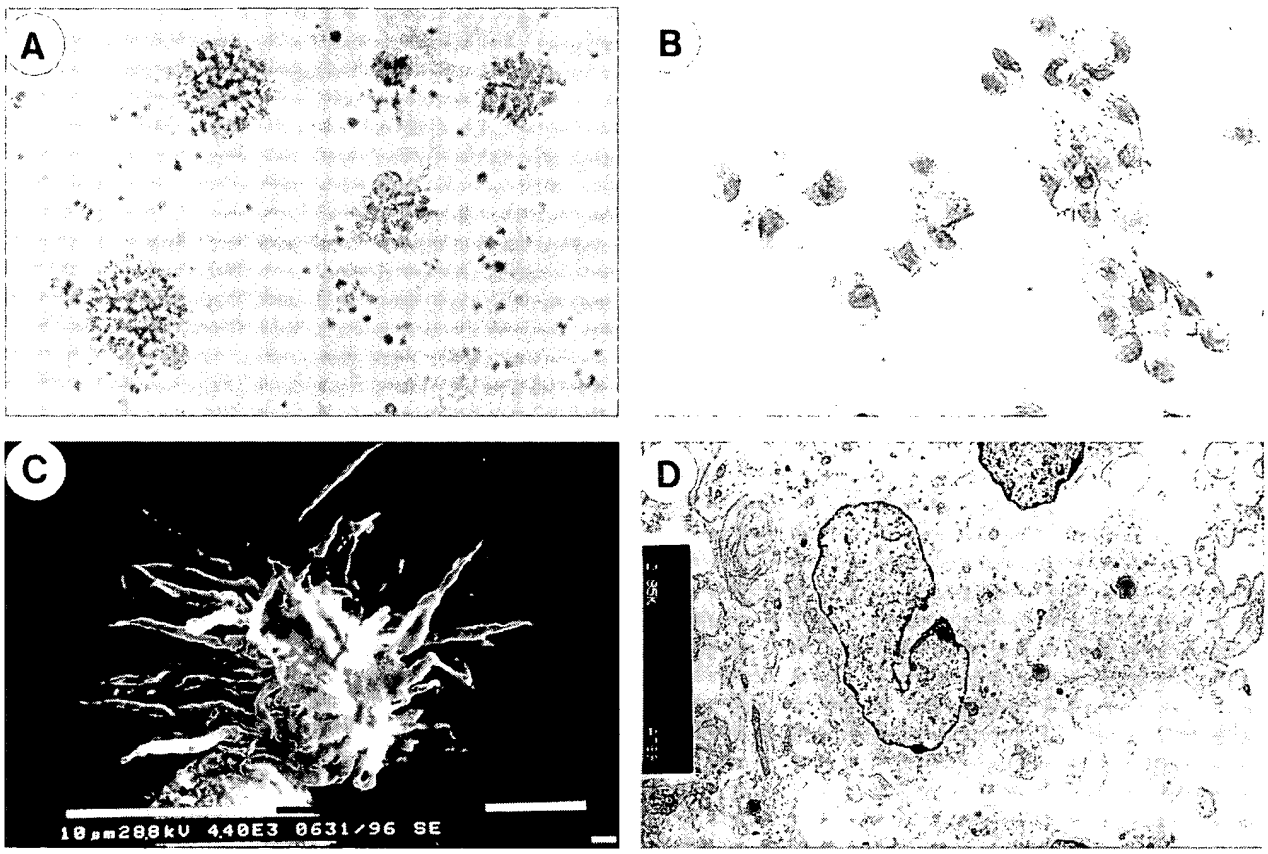

Figure 7. $CD4^+CD11c^-CD3^-$ plasmacytoid cells acquire the morphology of mature interdigitating dendritic cells after 6 d of culture with IL-3 and CD40-ligand. (*A*) Culture morphology; (*B*) Giemsa staining; (*C*) SEM; (*D*) TEM. Original magnification: (*A*) ×100; (*B*) ×400; (*C*) ×3,000; (*D*) ×6,000.

rophages (27, 34–36). This together with the fact that monocytes have the potential to differentiate into both macrophages and dendritic cells (11, 12, 37) suggests a myeloid DC developmental pathway. A critical cytokine for myeloid DC development was shown to be GM-CSF. Other factors such as TNFα, IL-4, SCF, and CD40-ligand were shown to enhance the myeloid DC maturation (23, 24). DC have also been generated from the most immature thymic T cell precursors or from bone marrow lymphoid progenitors, which could differentiate into T cells, B cells, and NK cells (28–32). These experiments suggest a lymphoid DC developmental pathway. Interestingly, GM-CSF does not appear to be required for DC generation from lymphoid precursors (31). In contrast, IL-3 seems to be one of the key cytokine required for lymphoid DC development (28–31). A recent experiment by Maraskovsky et al. (38) showed that Flt3L could potently enhance the development of both myeloid and lymphoid DCs in mice. The dependence of $CD4^+CD11c^-$ plasmacytoid cells on IL-3 but not GM-CSF to differentiate into DCs, which express relatively low levels of CD13 and CD33 myeloid antigens, suggests that these plasmacytoid cells may represent lymphoid-derived immediate DC precursors.

The capacity of CD40-ligand to promote the differentiation of $CD4^+CD11c^-Lin^-$ plasmacytoid cells into DC is consistent with the previous reports on the diverse effects of CD40-ligand on the maturation and function of DCs. Ligation of CD40 on dendritic cells was shown to trigger production of high levels of IL-12 (39, 40), enhance T cell stimulatory capacity (21, 41–43) and improve DC survival (21, 42, 43). A recent experiment showed that ligation of CD40 induced a subset of human $CD34^+$ hematopoietic progenitors to proliferate and differentiate into DC (44). Taken together, these data suggest that T cells play important roles in DC generation and maturation.

Tonsillar $CD4^+CD11c^-$ plasmacytoid cells share many similar features with the blood $CD4^+CD11c^-$ (15) or $CD33^{low}$ $CD14^{low}$ DC precursors (45). However, blood $CD4^+CD11c^-$ cells appear to survive better than tonsillar $CD4^+CD11c^-$ cells in culture medium (15, our unpublished observation), suggesting that the apoptosis program of these cells may be further triggered upon their arrival within tissues. Blood $CD4^+CD11c^-$ cells were shown earlier to differentiate into DCs in the presence of undefined factors within monocyte-conditioned medium (15). In our hands, these blood $CD4^+CD11c^-$ cells were found to become DCs in the presence of IL-3 and CD40-ligand (data not shown), as tonsillar $CD4^+CD11c^-$ cells. This strongly suggests that the tonsillar $CD4^+CD11c^-$ plasmacytoid cells are derived from blood $CD4^+CD11c^-$ cells.

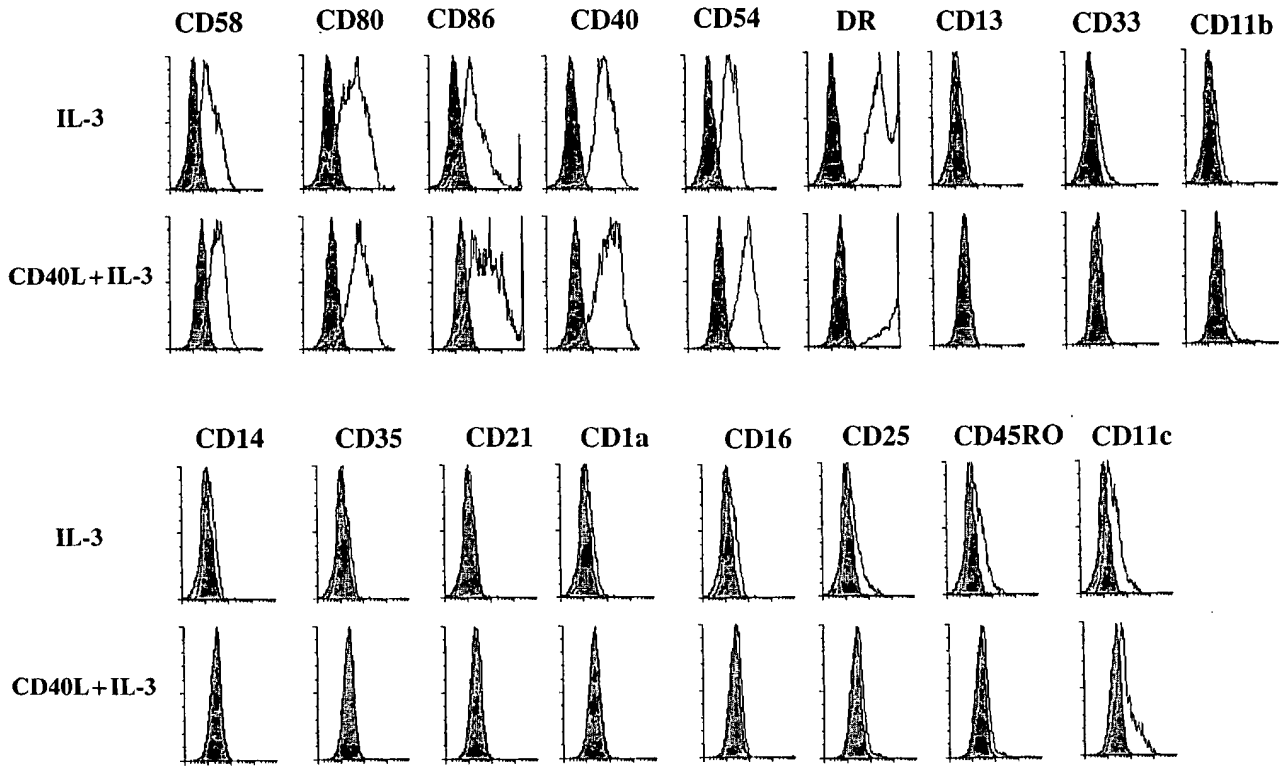

Figure 8. $CD4^+CD11c^-CD3^-$ plasmacytoid cells express high levels of costimulatory molecules, but few myeloid antigens, after 6 d of culture with IL-3 or IL-3+CD40-ligand. Filled histograms represent isotype matched controls, open histograms represent staining with specific antibodies. Data shown are one representative of six experiments.

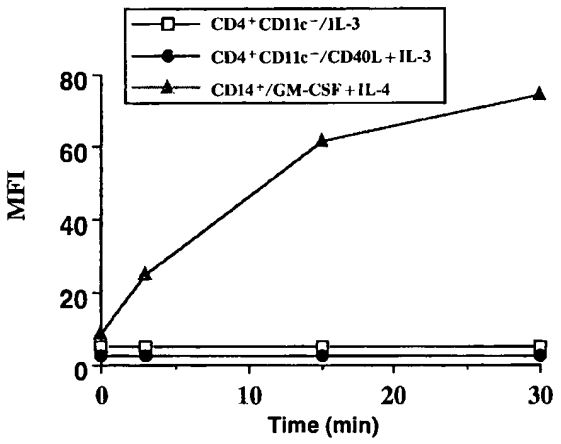

Figure 9. Dendritic cells derived from $CD4^+CD11c^-CD3^-$ plasmacytoid cells after 6 d of culture with IL-3+CD40-ligand did not uptake FITC-dextran. Methods are as described by Sallusto et al. (11). Time 0 represents cells that were incubated with FITC-dextran for 30 min at 4°C. After three washes, the FITC-dextran uptake by plasmacytoid cells derived DC cultured with IL-3 (*open squares*) or with IL-3 plus CD40-ligand (*solid circles*) and monocyte-derived DC (*solid triangles*) at 37°C were analyzed with a FACScan® flow cytometer. MFI, mean fluorescence intensity. Data shown are one representative of three experiments.

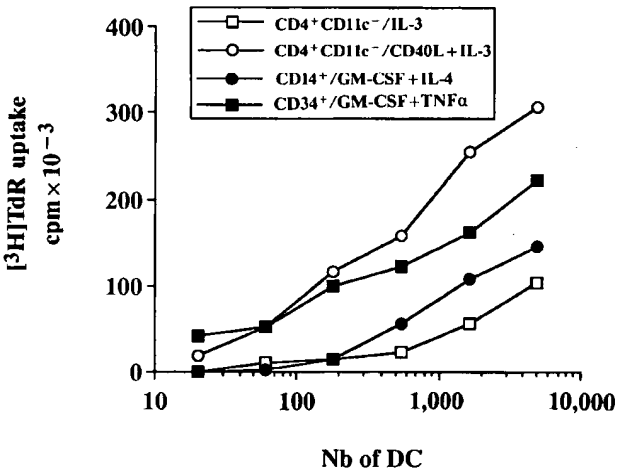

Figure 10. DC derived from $CD4^+CD11c^-CD3^-$ plasmacytoid cells induces strong proliferation of $CD4^+CD45RA^+$ naive T cells. Purified allogeneic blood $CD4^+CD45RA^+$ naive T cells were cocultured with different numbers of DC generated from: $CD14^+$ monocyte-derived DCs (*solid circle*), CD34+ progenitor-derived DCs (*solid square*), plasmacytoid cells after 6 d of culture with IL-3 (*open square*) and plasmacytoid cells after 6 d of culture with IL-3 and CD40L (*open circle*). After 5 d of DC-T cell cocultures, cells were pulsed with 1 μCi [^{3}H]thymidine for 8 h before harvesting and counting. Data shown are one representative of six experiments.

The increased accumulation of $CD4^+CD11c^-$ plasmacytoid cells in the draining lymph nodes of tumor patients suggests important immunological functions in these pathological conditions. They may be constantly produced in bone marrow and may migrate through blood to the T cell areas of the spleen by crossing the marginal zone sinuses or the T cell areas of lymph nodes or tonsils by crossing the HEV. There, they would undergo rapid apoptosis, unless rescued by T cell signals (IL-3+CD40-ligand) generated by on going antigen-driven T cell–dependent immune responses. Thus, the IL-3 and CD40-ligand–dependent DC development from plasmacytoid precursors may represent a unique peripheral regulatory pathway that can rapidly recruit large numbers of DC into immune responses.

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